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# Sucrose distribution in peach fruit during development and role of three distinct sucrose transporters

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# 1. Overall Introduction

## 1.1. Peach tree cultivation

The peach tree, *Prunus persica* (L.) Batsch, is the third most economically important fruit species in temperature regions after apple and pear (Byrne *et al*., 2012). During the last ten years peach harvesting has risen sharply, mainly due to the rapid increases occurred in China, the leader country which claim a production of about 50% of the total world through 2010, followed by Italy (7.5%), Spain (6%), the U.S.A. (6%) and Greece (3.5%) (Table 1). Over the last decade Italy occupies not only the second place in the world classification of major producers, but in the ranking of the larger exporters as well (Table 2).

**Table 1. World peach and nectarine production from 2000 to 2010. In the table are listed the twenty major world producers. Source: FAOSTAT, Statistical division of FAO (Food and Agriculture Organization of the United Nations [http://www.fao.org/statistics/en/]). All volumes are in tons.**

| | 2000 | 2001 | 2002 | 2003 | 2004 | 2005 | 2006 | 2007 | 2008 | 2009 | 2010 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **China** | 3827000 | 4561893 | 5230436 | 6148100 | 7010985 | 7624207 | 8214790 | 9051775 | 9534351 | 10141200 | 10800000 |
| **Italy** | 1655249 | 1679100 | 1586589 | 1175855 | 1710010 | 1693151 | 1664806 | 1630436 | 1589118 | 1691788 | 1590660 |
| **Spain** | 1129845 | 1082285 | 1275830 | 1270820 | 987574 | 1260878 | 1245527 | 1221073 | 1244291 | 1234886 | 1286456 |
| **U.S.A.** | 1412404 | 1341643 | 1422020 | 1341001 | 1429814 | 1301901 | 1132529 | 1279312 | 1304350 | 1200750 | 1254818 |
| **Greece** | 949943 | 943336 | 687000 | 228000 | 875544 | 864406 | 767938 | 816009 | 855200 | 821900 | 738400 |
| **Turkey** | 430000 | 460000 | 455000 | 470000 | 372000 | 510000 | 552775 | 539435 | 551906 | 547219 | 534903 |
| **Iran** | 350000 | 380000 | 385000 | 390000 | 400769 | 439771 | 370958 | 421304 | 574958 | 496130 | 496130 |
| **Egypt** | 240193 | 247300 | 339266 | 302667 | 360937 | 360000 | 427639 | 425273 | 399416 | 363209 | 273256 |
| **Chile** | 260000 | 290000 | 293000 | 304000 | 311000 | 311000 | 345000 | 370000 | 372000 | 388000 | 357000 |
| **France** | 480657 | 458135 | 455461 | 347218 | 396652 | 402646 | 394541 | 364947 | 299096,2 | 332049,8 | 311002 |
| **Argentina** | 209634 | 257768 | 211922 | 255785 | 272442 | 272500 | 260000 | 270000 | 308731 | 236948 | 318000 |
| **India** | 120000 | 150000 | 146176 | 155504 | 163851 | 173989 | 193342 | 211530 | 229557 | 231365 | 244000 |
| **Brazil** | 182460 | 222616 | 218292 | 220364 | 235720 | 235471 | 199719 | 185959 | 239149 | 216236 | 222402 |
| **Republic of Korea** | 170044 | 166275 | 187542 | 189413 | 200534 | 223701 | 193816 | 184497 | 189064 | 198317 | 138580 |
| **Algeria** | 59140 | 57674 | 65767 | 73964 | 80462 | 95059 | 117504 | 75174 | 118757 | 147400 | 156890 |
| **Mexico** | 147211 | 175752 | 197946 | 223883 | 201957 | 208185 | 222063 | 192261 | 190961 | 198085 | 227421 |
| **South Africa** | 223064 | 173868 | 189647 | 249290 | 183652 | 172631 | 166955 | 170005 | 182784 | 159444 | 164231 |
| **Japan** | 174600 | 175600 | 175100 | 157000 | 151900 | 174000 | 146300 | 150200 | 157300 | 150700 | 136700 |

Peach center of origin is recognized to be in Asia, in particular it was domesticated in China about 4000 years ago (Faust and Timon, 1995). Peach dispersal around the world has been widely described (Figure 1). It started about 3000 years ago, when it moved from its center of origin throughout temperate and subtropical Asia regions. Afterwards, it spread to Japan and to Persia (present day Iran) along silk trading routes, more than 2000 years ago, and from there it was diffused by Greeks, and especially Romans throughout Europe and northern Africa, starting from 300-400 BC. In fact, the name “persica” was utilized from Romans because they wrongly thought

peaches originated from Persia. It reached the new world with settlers during the sixteenth and seventeenth centuries, in particular the northern Florida coast of North America, and South America by Spanish and Portuguese explorers, respectively, and then distributed in a wide range of regions of America by Native Americans and colonizers (Byrne *et al*., 2012).

**Table 2. World peach and nectarine exports from 2000 to 2010. In the table are listed the ten major world exporters. Source: FAOSTAT, Statistical division of FAO (Food and Agriculture Organization of the United Nations [http://www.fao.org/statistics/en/]). All volumes are in tons.**

| | 2000 | 2001 | 2002 | 2003 | 2004 | 2005 | 2006 | 2007 | 2008 | 2009 | 2010 | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Spain** | 261573 | 251701 | 342913 | 363369 | 259876 | 394124 | 529579 | 472640 | 546981 | 574307 | 585294 | 4582357 |
| **Italy** | 407869 | 407333 | 394411 | 327032 | 406046 | 421674 | 357147 | 369985 | 326761 | 357908 | 359597 | 4135763 |
| **U.S.A.** | 204490 | 242137 | 213243 | 195689 | 167421 | 175798 | 134609 | 175922 | 192681 | 142265 | 171450 | 2015705 |
| **Chile** | 86093 | 107510 | 0 | 130782 | 121383 | 117402 | 105464 | 106165 | 117078 | 108374 | 104542 | 1104793 |
| **Greece** | 129453 | 155152 | 99998 | 13873 | 97979 | 102790 | 80829 | 82728 | 102300 | 79350 | 110580 | 1055032 |
| **Turkey** | 14583 | 23681 | 27580 | 44228 | 20152 | 39303 | 39121 | 18994 | 42931 | 32319 | 41390 | 344282 |
| **China** | 9463 | 3819 | 14198 | 19366 | 15729 | 17995 | 21574 | 25369 | 27299 | 41834 | 28636 | 225282 |
| **Poland** | 0 | 20829 | 135 | 197 | 14064 | 9945 | 11842 | 26085 | 18720 | 20989 | 23433 | 146239 |
| **Jordan** | 1660 | 2053 | 2823 | 3502 | 3944 | 6090 | 9831 | 12889 | 18770 | 25206 | 30629 | 117397 |
| **Lithuania** | 130 | 221 | 245 | 222 | 364 | 1331 | 3716 | 7029 | 14769 | 17102 | 23718 | 68847 |

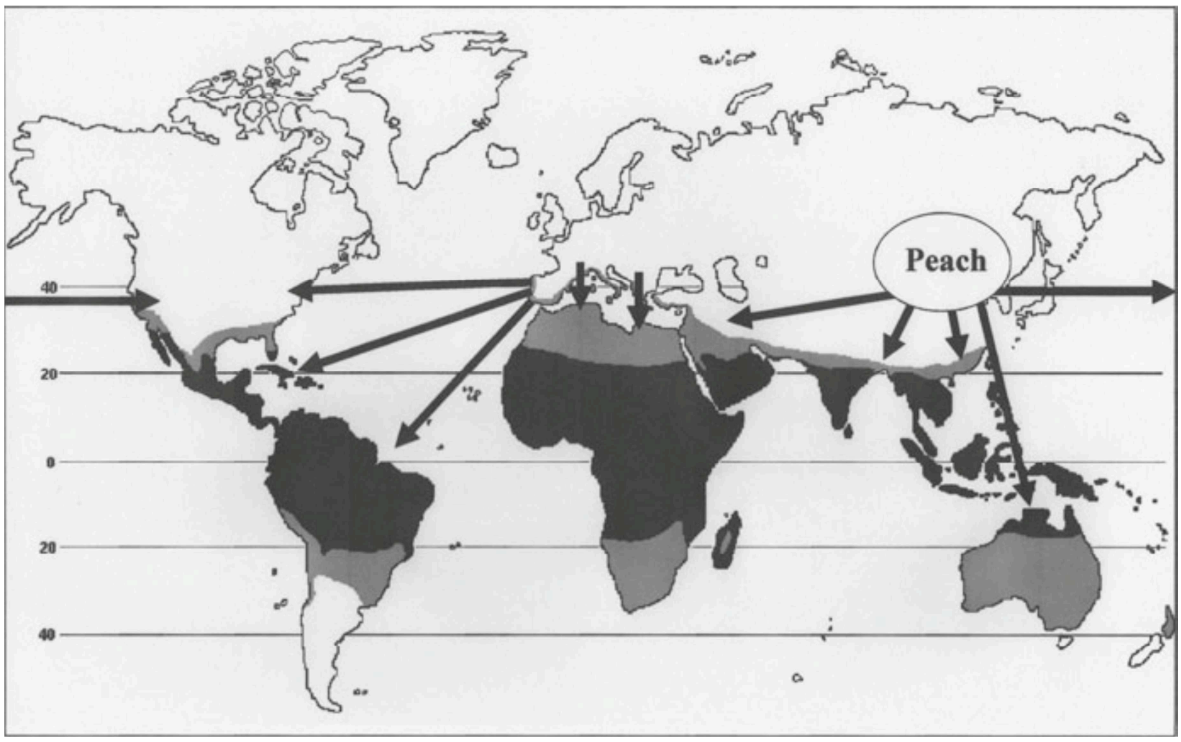


**Figure 1. Origin and dissemination of peach. The areas highlighted in white, grey and black indicate cold, medium to low chill, and tropical zones, respectively (from Byrne *et al*., 2012).**

Peach belongs to the Rosaceae family, more in detail to subfamily Prunoideae, genus *Prunus* (L.), and subgenus *Amygdalus*. The family of Rosaceae encompasses over 100 genera and 3000 species,

including the genera *Malus*, *Pyrus* and *Prunus*, cultivated for their fruits (Shulaev *et al*., 2008), appreciated for a great variety of traits such as textures, flavors and colors, but also for their benefits on human health and nutrition due to metabolites like flavonoids, anthocyanins, and phenolics (Swanson, 1998). Together with apple (*Malus domestica*) and strawberry (*Fragraria vesca*), peach has been used as a model plant for studying genomics in the Rosaceae (Shulaev *et al*., 2008). In fact, is a diploid species (2n=16) with a small genome size, only about twice then *Arabidopsis*, has a relative short juvenile period (2-3 years until flowering), and it is self-compatible.

Besides peach, the Prunus genus includes other important marketed species, such as almond (*Prunus amygdalus* Batsch), apricot (*Prunus armeniaca* L.), sour cherry (*Prunus cerasus* L.), sweet cherry (*Prunus avium* L.) or European plum (*Prunus domestica* L.) (Uematsu *et al*., 1991). All of these species produce a fleshy fruit, called drupe, and are collectively referred to as "stone fruits" (Figure 2).

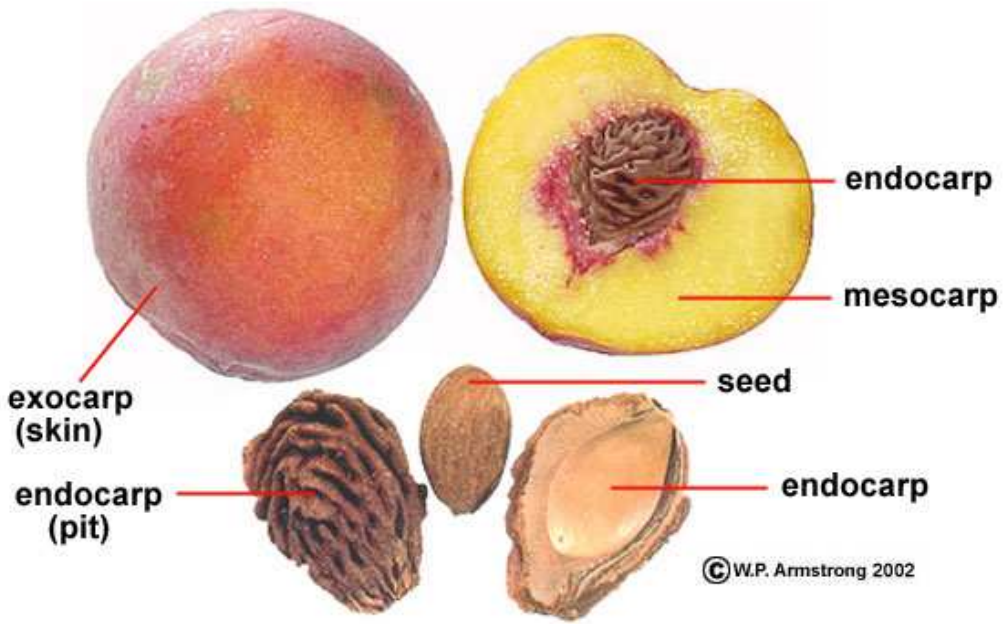


**Figure 2. Different parts that constitute a typical stone fruit (from http://waynesword.palomar.edu/termfr4.htm).**

Peach fruits are appreciated for different quality attributes, such as sweetness, size, flesh firmness (melting or non-melting), flesh color (from white to yellow, to dark red, with variation in tonalities), volatiles compounds production, antioxidants content (Infante *et al*., 2008).

## 1.2. Growth and development of peach fruit

In botanical terms, fruit, is defined as the specialized organ produced only by flowering plants (Angiosperm), that typically starts to form after ovule fertilization. It originates from the development and enlargement of the ovary, while fertilized ovule develops into a seed. Its function is to create a suitable environment for seeds development and often to aid their dispersal at mature stage (Seymour *et al*., 2008). In some instances, fruit develops without fertilization (or even if this occurred is followed by abortion), and hence without seed formation. This phenomenon, called parthenocarpy, spontaneously occurs in several plant species such as orange, persimmon, banana, grape, or pineapple (Gustafson, 1942).

Fruit growth relies on two distinct mechanisms, cell division and cell enlargement, that occur at different developmental stages. The increase in cell number takes place in the earlier stages of fruit development, when the increment in fruit size and weight is not significant. The number of cells which forms the fruit is set upon the completion of cell division. The end of this process varies according to species, for example, in apple it terminates in about 4-5 weeks after bloom and represents 20% of the total growth period (Bain and Robertson, 1951), but in some cases the increase in cell number lasts until harvest, as in avocado fruit (Schroeder, 1958). The completion of cell division is followed by a period of rapid increase in fruit size as a consequence of the gradual enlargement of parenchyma cells. The increase in cell dimension is mainly due to vacuolar enlargement, as much as, in mature fruit, vacuole occupies most of the cell volume. During this phase of fruit growth, several compounds are stored inside the vacuole, such as sugars, organic acids and also pigments (Sinha *et al*., 2012).

In *Prunus persica*, fruit growth is strictly connected to embryogenesis (Chalmers and Van den Ende, 1977; Bonghi *et al*., 2011). Like the other stone fruits, peach exhibits a particular growth pattern that can be described as a double sigmoid in which four stages can be distinguished (Figure 3) (Bonghi *et al*., 2011). The early period of development (stage I) is characterized by a rapid growth, primarily due to the active cell division, even if during the second half some cells enlarge, and a number of intracellular spaces develops. Cell division terminates with the end of this phase. Concomitant to the first increase in fruit size, seed also grows and reaches its maximum length. During the second phase (stage II), called also lag-phase, the endocarp hardens to form the stone (process known as “pit hardening”) along with a relatively slow down of fruit growth rate. The embryo starts to develop, whereas the third period of development (stage III) is

characterized by the enlargement of mesocarp cells that generate the second exponential growth phase. Maturation is completed and followed by ripening in the final stage (stage IV) (Ognjanov *et al*. 1995; Bonghi *et al*., 2011).

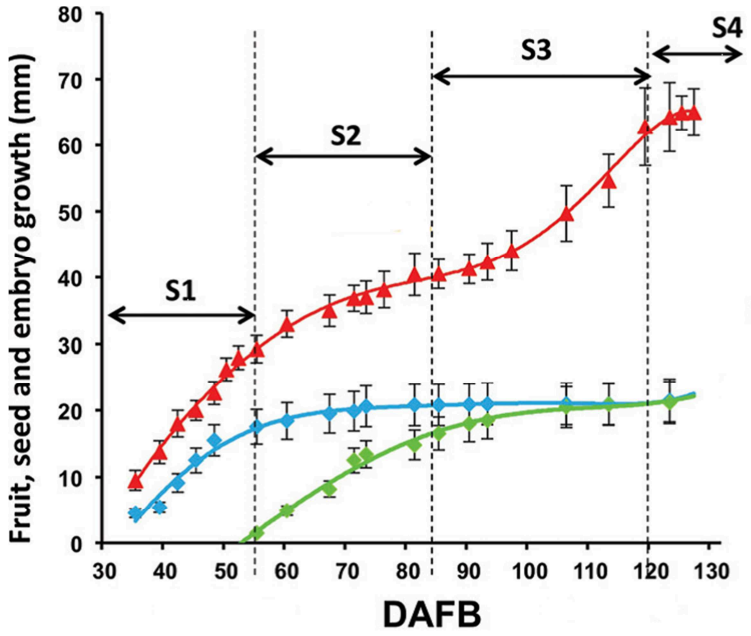


**Figure 3. Fruit (red), seed (blue) and embryo (green) growth pattern, obtained by measuring cross diameter (for fruit), or length (for seed and embryo) during development (expressed as day after full bloom [DAFB]) (from Bonghi *et al*., 2011).**

Peach cultivars characterized by a different time of ripening exhibit a double sigmoid growth pattern with an alteration in the duration of the second phase of development. More precisely, in early maturing cultivars the lag phase is very short and it may last only few days, whereas cultivars described as late-maturing showed a very long growth slowdown, up to two months (Ognjanov *et al*. 1995; Bonghi *et al*., 2011).

## 1.3. Carbohydrates functions in fruits

Fruit quality, that plays an essential role in consumer satisfaction and consequently has implications on further demand, has been defined by Kader (1999) as the combination of attributes, properties, or characteristics that give each commodity value in terms of human food. This concept includes several external and internal desirable characteristics that describe the excellence or superiority of a given product. External aspects encompass everything that can be evaluated visibly, such as appearance, uniformity, size, shape, ripeness, and are the first yardstick by the consumers, while internal properties include nutritive values, flavor, aroma, texture and

absence of biotic and non-biotic contaminants that are equally important to the consumers (Kader, 1999).

The amount of sugars is one of the attributes that define fruit quality, since, together with the quantity of organic acids, bitter compounds such as alkaloids or flavonoids, salts, and aroma volatiles (Colaric *et al*., 2005; Song and Forney, 2008; Seymour *et al*., 2013), determines fruit flavor. Fruit sweetness is correlated not only to the total sugar concentration, but also to the sugars composition, since carbohydrates differ in their relative sweetness (Kulp *et al*., 1991; Robertson *et al*., 1992). In fact, if sucrose is rated 1.0 in terms of sweetness, fructose is rated about 1.75, glucose 0.75 and sorbitol 0.6 (Pangborn, 1963; Doty, 1976).

Beside this function, carbohydrates play an important role in fruit growth and development, being the main source of energy for the metabolism. Furthermore, they have a function in cell enlargement, necessary for the increase of fruit size. In fact, sugars are stored into the vacuole as a carbon reserve. The high carbohydrates accumulation stimulates influx of water into the storage organelle. The consequence of this process is an increase of turgor pressure against the cell wall that lead to cellular enlargement, and hence fruit growth (Yamaki, 1984). For instance, quantification of sugars content in different compartment of mature apple tissues, i.e. isolated vacuoles and apoplast spaces, revealed that carbohydrates accumulation forms about 11 atm of turgor pressure (Yamaki and Ino, 1992).

Finally, several observations have highlighted the role of sugars as signaling molecules for the coordination between photosynthesis and energy demand (Hanson and Smeekens, 2009). This role has been attributed to different types of sugars, i.e. glucose, fructose and more recently sucrose and trehalose-6-phosphate (Rolland *et al*., 2006).

## 1.4. Source and sink organs

Most of the carbohydrates are imported into fruits and used either to sustain growth or stored as reserve. Fruits are, in fact, primarily heterotrophic organs (sink organs). Generally, they contain chlorophyll only in the early period of growth, during this phase they can fix carbon when exposed to sunlight (Blanke and Lenz, 1989; Carrara *et al*., 2001). In some cases, like avocado, chlorophyll persists during the whole period of development, but the contribution of carbohydrates derived from own fixed carbon is not sufficient to support growth and development (Whiley *et al*., 1992). Estimation of photosynthetic rate in the reproductive organs of different plant species has indicated variation in the efficiency of photosynthesis; for example the contribution was found to

be 5-9% for peach (Pavel and DeJong, 1993), 12% for pepper (Steer and Pearson, 1976), and 10-15% for tomato (Tanaka *et al*., 1974). Therefore, the growth and development of fruits are strictly dependent on the supply of carbohydrates from other parts of plant (source organs).

Source organs are usually photosynthetically active, and produce an excess of carbohydrates compared to their own needs, therefore they are net exporters of photoassimilates (mainly mature leaves, but also storage organs during exporting phase of development, like tubers). Sink organs, referred as net importers of fixed carbon, can be further divided into at least two different classes: utilization and storage sinks. Utilization sinks are characterized by high mitotic activity and extension growth, such as developing leaves, growing roots and shoot apices. Therefore, in these tissues, most of the imported assimilate are used for catabolism and only a small amount is temporarily stored. Storage sinks are the organs where the imported carbohydrates are deposited in the form of storage compounds (e.g. carbohydrates, fatty acids, or proteins) (Sonnewald and Willmitzer, 1992). Among this group, the major carbohydrate reserves are soluble sugars (sucrose, glucose or fructose) and starch. For example, in sugar beet tap root about 70% of dry matter accumulated is sucrose (Milford, 1973); while more than 80% of wheat grain endosperm consists of starch (Jennings and Morton, 1963). Regarding fruit tree, some plant species accumulate one or more soluble sugars into storage sinks, for instance grape berry accumulates glucose and fructose (Davies and Robinson, 1996), while other sinks collect both soluble sugars and starch, in variable amounts according to plant species. For example, apple fruit uses fructose as the main carbohydrate reserve, but during fruit growth accumulates also starch, that is degraded during the last phase of development to enrich the sucrose reserve stored in the vacuole (Li *et al*., 2012). Kiwifruit accumulates large amounts of starch throughout development, about 50% of the total dry matter towards the end of fruit growth, and store only minor amount of soluble sugars (Seager and Haslemore, 1993; Nardozza *et al*., 2011).

## 1.5. Phloem transport

The surplus of photoassimilates produced in source organs are distributed towards sink organs through the phloem, the long-distance transport system. This vascular tissue can be divided at least in three parts, in relation to organ localization, and task: *collection phloem* in source organs, in which photoassimilates move from mesophyll cells into phloem strands, *transport phloem* along the path that connects source and sink organs, and *release phloem* in sink organs where compounds are distributed into parenchyma cells (van Bel, 1996). Contrary to the collection and

release regions, that generate a one-way traffic of solutes into and out of phloem, respectively, the transport phloem have a dual function: carrying photoassimilates to terminal sink, and concurrently supplying the heterotrophic tissues along the phloem pathway through a leak and retrieval of photoassimilates during transport (van Bel, 2003).

The phloem of angiosperm is composed of several types of cells that are closely associated with the xylem within vascular bundles: the phloem sap conducting sieve elements (SE), the companion cells (CCs), and the phloem parenchyma cells. The SE cells are highly modified to create a low-resistance pathway. In fact, during maturation, sieve elements extend longitudinally, the endoplasmic reticulum is reorganized, the plastids and mitochondria undergo several changes, while nucleus, vacuole and ribosomes are disassembled and disappear. At the same time, changes occur in the cell wall, and the specialized sieve area pores are developed (Cronshaw, 1981). The companion cells are characterized by prominent connections with the sieve elements through a modified plasmodesmatal complex, known as plasmodesmata pore unit, dense cytoplasm and numerous mitochondria. These are essential for the survival of SEs via plasmodesmatal links through the supply of energy and proteins (Lucas *et al*., 1993). The SE forms a functional complex with its associated CCs, called SE/CC complex (SECCC) that is connected to surrounding phloem parenchyma cells and other cell types by plasmodesmata, primarily via the CC (Turgeon and Wolf, 2009). The conductivity and the frequency of these plasmodesmata change greatly according to species, tissue type and stage of development (Gamalei, 1991; Roberts *et al*., 2001).

Through the phloem sap, that moves in sieve elements with rates of about 3 m for h (Kühn, 2003), several compounds are transported: amino acids with a concentration in the range of 200-400 mM (Dinant *et al*., 2010); proteins that are detectable in variable quantity, from 0.1 to 30 mg $mL^{-1}$ (Fisher *et al*., 1992), in some cases higher, for example in pumpkin (*Cucurbita maxima*) has been reported a value of 100 mg mL-1 (Richardson *et al*., 1982); inorganic ions, such as potassium, can accumulate up to 125 mM (Deeken *et al*., 2002); and, in low amounts, other compounds including organic acids, hormones and secondary products (Öpik and Rolfe, 2005). The most abundant components of phloem sap are carbohydrates. Their concentration can reaches values between 300 and 1000 mM. In the majority of species, sucrose is the main form of reduced carbon transported in sieve elements, but in some plant species other types of non-reducing sugars can be find such as the raffinose-family oligosaccharides or sugars alcohol (sorbitol, dulcitol or mannitol) (Öpik and Rolfe, 2005). For example, the analysis of carbohydrate concentration and composition of phloem sap carried out in species belonging to the Rosaceous family, such as apple

and pear, has shown that they generally transport both sucrose and sorbitol, and generally sorbitol is present in higher concentration than sucrose (Loescher, 1987). Within this family there are some exceptions. For instance, strawberry that accumulate glucose, fructose and sucrose in mature fruit, translocates preferentially sucrose, although low sorbitol concentration have been measured in some varieties (Sutsawat *et al*., 2008). The sugar composition of phloem sap has also been analyzed in *Prunus persica* and revealed the presence of both sucrose and sorbitol (Moing *et al*., 1992). The quantification of carbohydrate levels in phloem sap from mature leaves of different peach cultivars has revealed variability in sorbitol:sucrose ratio depending on genetic, and geographic origin (Japanese, American and European cultivars) (Escobar-Gutierrez and Gaudillère, 1994). Moreover, the ratio is also related to growth stage and organ analyzed (Lo Bianco *et al*., 1999). Actually, it's not still really clear if peach preferentially use sorbitol or sucrose as the primary source of transported carbohydrate (Layne and Bassi, 2008). Some authors have postulated a preferential utilization of sorbitol in vegetative sink tissues close to the source, such as young sink leaves and cambium (Moing *et al*., 1992), and a major role of sucrose in fruit growth (Lo Bianco *et al*., 1999).

The process of phloem transport begins with the phloem loading in the source tissues, a step in which solutes, usually sucrose, move from parenchyma cells to SECCC. There are three accepted strategies of sucrose loading: symplasmic with or without polymer trapping, and apoplasmic (Rennie and Turgeon, 2009). The symplasmic loading occurs when a functional symplasmic continuity exists between the SECCC and adjacent cells, given by the presence of numerous plasmodesmata and is driven by decreasing sugar concentration from the mesophyll to the phloem strands (Van Bel, 1993). In some plants, the process is driven by passive diffusion (Reidel *et al*., 2009), while, in other species, sucrose spreads into specialized companion cells, known as intermediary cells, and is converted to raffinose-family oligosaccharides (RFOs). Since RFOs are larger than sucrose, they are apparently unable to flow back through the small plasmodesmata localize in the interface between intermediary and mesophyll cells, but they can diffuse across the larger plasmodesmata between intermediary cells and sieve tubes down their concentration gradients. This process is known as polymer-trapping (Turgeon, 1991). In apoplasmic loaders, the numbers of plasmodesmata between SECCC and surrounding cells is very low or null. Since sucrose cannot reach the phloem symplasmically, it has to be release from mesophyll cells into the apoplast, then actively taken up by sucrose transporters localized in the plasma membrane of phloem cells (Dinant and Lemoine, 2010). When reached the sink tissues, photoassimilates exit

from phloem elements, through a process known as phloem unloading, and move into the post-phloem pathway, where they are used to sustain growth and development or as storage carbohydrates (Oparka and van Bel, 1992). The unloading pathway takes place, also in this case, either through symplasmic or apoplasmic route. In the first instance, the movement of sugars, in particular sucrose, occurs via plasmodesmata, and can be regulated by changes in the structure and number of plasmodesmata or by alteration of their conductivity (Herbers and Sonnewald, 1998; Baluska *et al*., 2001); in the second case, sucrose moves across the plasma membrane of SECCC, through the involvement of sugar transporters, to intercellular spaces (the apoplasmic pathway). Sucrose is first unloaded in the apoplast, and then taken up via two different mechanisms: directly by sucrose transporters localize to plasma membrane or as monosaccharide by hexose transporters after hydrolysis into glucose and fructose by cell wall-bound invertase (Lalonde *et al*., 1999). The unloading of phloem in species that transport RFOs in addition to sucrose has received less attention. Recently, the phloem transport has been analyzed in cucumber fruit (*Cucumis sativus* L.), a typical stachyose-transporting species, and an integrated model of soluble sugars unloading has been proposed. The authors hypothesized the presence of *trans*-membrane specific transporters responsible for the unloading of RFOs. Once RFOs reach apoplast, they may be hydrolyzed in sucrose or hexoses and therefore uptaken as mono- or disaccharides. However, to date RFO transporters have not been identified at the molecular level in any of the higher plants (Hu L. *et al*., 2011).

The processes of phloem loading and unloading create a hydrostatic pressure gradient along phloem strands that generates the driving force for nutrient movement from source to sink organs (Munch, 1930).

## 1.6. Phloem unloading and sink strength

The growth and development of sink organs is highly dependent on the supply of carbohydrates from phloem. The quantity of sugars that moves from source to sink through the phloem pathway can be determined by either a source limitation or a sink limitation (Patrick, 1988; Morandi and Grappadelli, 2009). The source limitation occurs when the source organs are not able to supply sufficient resources due to a low photosynthetic rate or an inefficient phloem loading, while sink limitation is linked to the sink strength, defined as the competitive ability of an organ to import photoassimilates (Herbers and Sonnewald, 1998). Sink strength is determine by both sink size, i.e. the number of cells that forms a given sink organ, and sink activity that comprises three metabolic

processes: the movement of assimilate from phloem within the recipient sink cells (phloem unloading, post-phloem transport, and retrieval into parenchyma cells), their utilization in the metabolic processes, and their storage (Yamaki, 2010).

In the whole plant there are several types of sink regions (seeds, fruits, sink leaves, roots, and apices) that draw photosynthates from the same pool of nutrients; hence they are in competition with each other for the available reserves. Organs that have high sink strength are able to attract more photoassimilates compared to those with low sink strength. Some authors have classified sinks according to the ordering of preference for the sinks supply (Minchin and Thorpe, 1996), for instance seeds have been describe as high priority sink (Cannell, 1985).

It is now well accepted that processes of phloem unloading and post-phloem transport, events that link phloem transport with sink metabolism and/or compartmentation, play a key role in determining sink strength and, consequently, in the partitioning of photoassimilates, impacting on crop yield and plant productivity (Fisher and Oparka, 1996; Patrick, 1997). Sugars can efflux and reach sink cells either by simple diffusion through plasmodesmata into vascular parenchyma elements or directly across the plasmalemma into the apoplast by the involvement of plasma membrane proteins. Both pathways can operate concurrently, but usually one route is preferentially adopted depending on species, sink types, sink development, sink function and also environmental conditions (Oparka *et al*., 1999; Roberts *et al*., 1997; Viola *et al*. 2001; Itaya *et al*. 2002).

Different approaches have been implemented to examine the connection between SE/CC complexes and surrounding cells in several sink organs, such as anatomical investigation by means of electron microscopy, that allows to calculate the plasmodesmata abundance between phloem and parenchyma cells and to observe their structure (Oparka, 1986; Zhang *et al*., 2004; Zhang *et al*., 2006; Nie *et al*., 2010), or physiological investigation, through the analysis of phloem functionality that can be achieved by the observation of tracers transport (i.e. carboxyfluorescein, [$^{14}$C] sugar or tobacco mosaic virus movement protein fused to green fluorescent protein) (Roberts *et al*., 2001; Imlau *et al*., 1999; Damon *et al*., 1988; Zhang *et al*., 2006; Nie *et al*., 2010).

## 1.7. Sugar transporters

When the symplasmic continuity between SECCC and parenchyma cells is interrupted, the movement of sugars can occur only across the plasma membrane, through the involvement of membrane proteins. In this context, sugars transporters assume a crucial role for the cell-to-cell

and long-distance distribution of carbohydrate throughout the plant. Many genes encoding sugar transporters have been identified and classified into eight large groups: sucrose transporters (SUC/SUT), sugar transport proteins (STP), early response to dehydration-like (EDR6-like), plastidic glucose transporter/Supressor of G protein beta1 (pGlucT/SBG1), inositol or cyclic polyol transporters (INT), linear polyol transporters (PMT, formerly PLT), tonoplastic monosaccharide transporters (TMT), and vacuolar glucose transporters (VGT) (Shiratake, 2008). Excluding sucrose transporters, all the other seven groups belong to the Monosaccharide Transporter (MST or MST-like) family (Figure 4) that in Arabidopsis comprises 53 genes, in barrel medic (*Medicago trunculata*) 58 genes, in grapevine (*Vitis vinifera*) 59 genes, and in rice (*Oryza sativa*) 65 genes (Doidy *et al*., 2012). All of these transporters have a high similarity in the membrane topology, and are predicted to share a common structure with twelve membrane spanning domains that interact to form a central pore allowing the movement of sugar across biological membranes (Büttner and Sauer, 2000). Moreover, members grouped within the same sub-family have a quite high homology degree, ranging from 42% to 96%, but when the comparison is made between transporters belonging to different group, the similarity significantly drops supporting the classification into distinct families (Büttner, 2007). Many monosaccharide transporters belonging to different groups are able to carry similar sugars, moreover, they can transport more than one monosaccharide, even if they have a high affinity for some carbohydrates (Slewinski, 2011).

Recently, a novel and different group of transporters, called SWEET proteins, have been identified based on their capacity to transport glucose across a membrane. They cluster into a distinct family (Chen *et al*., 2010), particularly, in the genome of *Arabidopsis*, *Medicago trunculata* and *Oryza sativa* 17 genes, 15 genes and 21 genes have been found, respectively (Doidy *et al*., 2012). SWEETs are small proteins showing seven transmembrane helices which form a single pore that allows the diffusion of glucose along a concentration gradient. Their transport activity is not influenced by changes in pH, and hence doesn't appear to require a proton gradient. Recent studies have suggested that they function as facilitators mediating both the influx and the efflux of sugars into and out of cells (Chen *et al*., 2010). Some members of these family showed limited or no ability to transport glucose, such as AtSWEET11 and AtSWEET12, but displayed the capability to mediate sucrose flux across biological membranes (Chen *et al*., 2012).

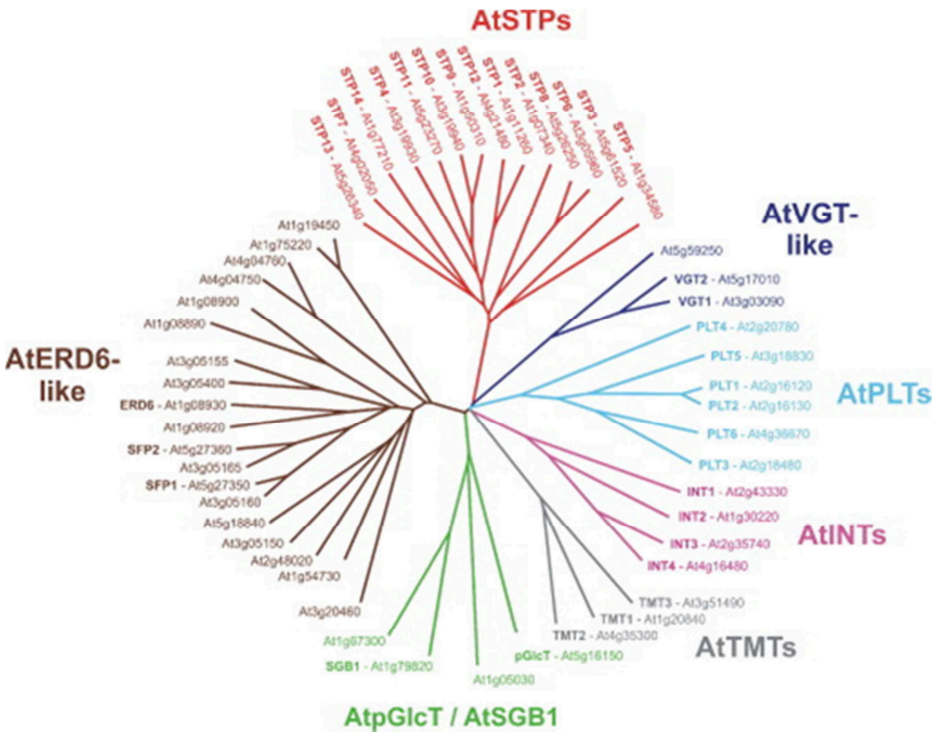


**Figure 4. Phylogenetic tree of the monosaccharide transporter(-like) gene family in Arabidopsis from Büttner (2007).**

## 1.8. Sucrose transporters

Sucrose transporters are important $H^+$-symporter proteins that utilize the electrochemical gradient, generated by primary active transport proteins, to move the sucrose across a membrane against a concentration gradient, and have an essential role when the symplasmic continuity between SECCC and parenchyma cells is interrupted. The first sucrose transporter cDNA (*SoSUT1*) was identified in spinach (*Spinacia oleracea*), by functional analysis in yeast (Riesmeier *et al.*, 1992).

Since the first sucrose transporter cDNA discovered, and thanks to the completion of the genome sequence of several species, many other genes have been identified and characterized in a wide variety of plants, both dicot and monocot. All plant sucrose transporters are encoded by the SUcrose Transporter (SUT) gene family, also called SUC (SUcrose Carriers) genes, that belong to the major facilitator superfamily (MFS) (Marger and Saier, 1993) and in particular to the Gycoside-Pentoside-Hexuronide (GPH) cation symporter family, a distinct subfamily of the MFS (Marger and Saier, 1993; Saier *et al*., 2006). Today, the MFS includes more than 10000 members, transporting a diverse variety of substrate (Pao *et al*., 1998; Saier *et al*., 2006). The most widely studied MFS protein is the lactose permease of *E. coli* (LacY), which transports lactose and $H^+$ into the cell at a ratio of 1:1 (West and Mitchell, 1973). The *Arabidopsis* genome contains the largest SUT gene family described to date, with nine SUT homologs, two of which, *AtSUC6* and *AtSUC7*, are

characterized as pseudogenes (Sauer *et al*., 2004), whereas there are five genes identified in *Oryza sativa* (Aoki *et al*., 2003). Regarding the classification, various authors have used different convention in the nomenclature (Ayre, 2011). According to the latest classification (Kühn and Grof, 2010), SUTs family is divided into five major clades, from SUT1 to SUT5. Only two of them contain members from both mono- and dicotyledonous plants, SUT2 and SUT4 subfamily, while SUT1 clade is dicot specific, and members of SUT3 and SUT5 clades are monocot specific (Figure 5).

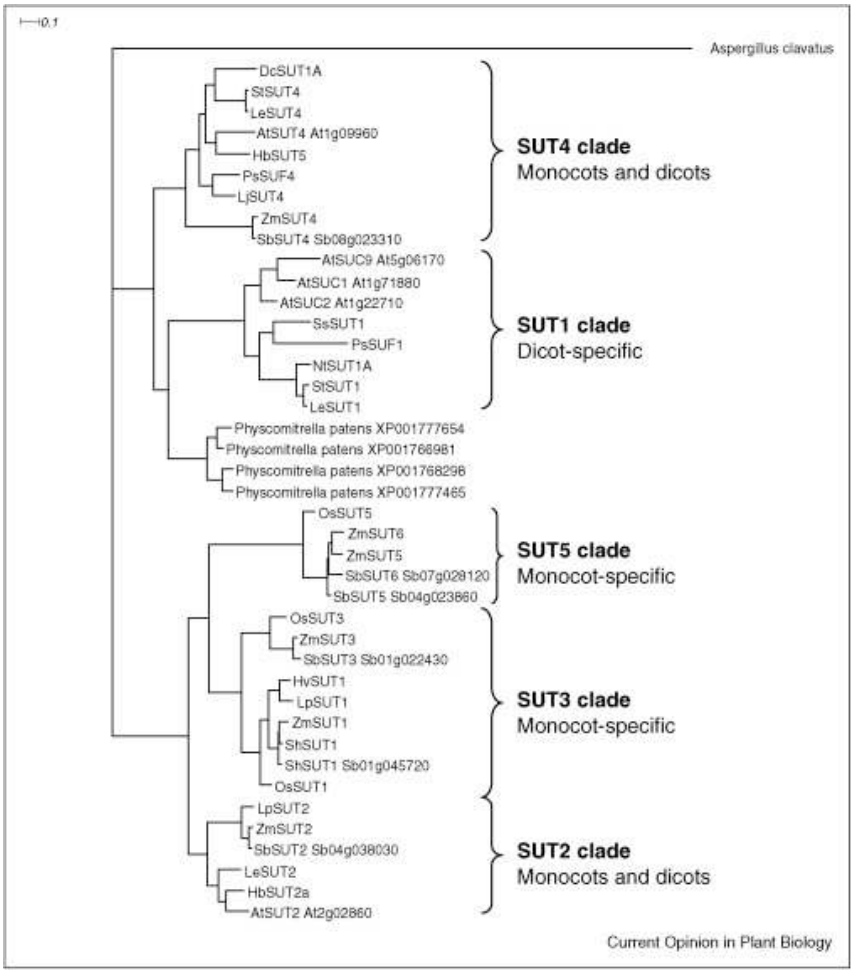


**Figure 5. Classification of sucrose transporters into five distinct clades from Kühn and Grof (2010).**

## 1.9. Sucrose transporters structure

The SUT genes encode highly hydrophobic membrane-localized proteins. Structural information about these proteins is limited to sequence homologies with members of the major facilitator superfamily (MFS) found in bacterial and animals; for example, the lactose permease from *E. coli* is one of the best characterized protein representative of the MFS. It was often used as a template to predict tertiary structure of sucrose transporters because its crystal structure at high-resolution

is available (Zuniga *et al*., 2001; Abramson *et al*., 2003a; Vardy *et al*., 2004). The lactose permease is formed by two halves consisting of six transmembrane domains, binding each other, that show a high degree of symmetry (Figure 6). This observation has led some authors to hypothesize that the two domains have the same genetic origin, although the comparison of sequence reveals a low level of homology. The protein contains a unique substrate binding site that is localized at the interface of the two halves. A working model has been proposed in which the sugar-binding site is alternatively open to either side of the membrane (Abramson *et al*., 2003b).

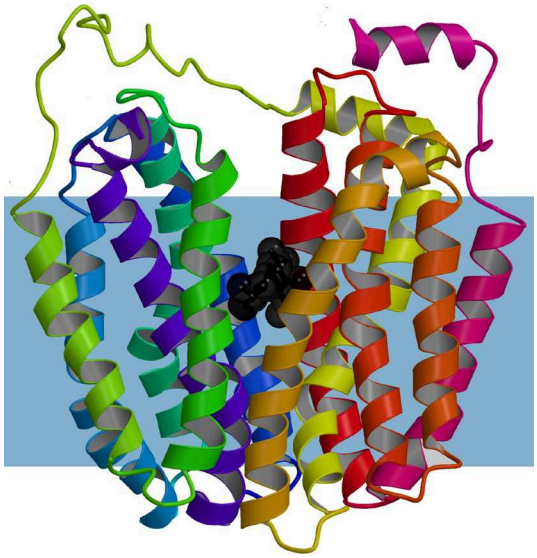

**Figure 6. Structure of the lactose permease of Escherichia coli (from Abramson *et al*., 2003).**

Consistent with the members of MFS, hydropathy analysis with plant sucrose transporters predicted twelve transmembrane-spanning domains connected by eleven hydrophilic loops (Figure 7) (Riesmeier *et al*., 1992; Sauer and Stolz, 1994). As for the lactose permease, some authors have speculated that the 12 transmembrane helices are the result of gene duplication and fusion of two ancestral transporters with only six transmembrane domains (Saier, 2002). In the MFS, the two halves composed of six transmembrane domains are linked by a long central cytoplasmic loop. Site-directed mutagenesis has shown that residues inside this loop have no essential function in the transport activity (Frillingos *et al*., 1998), but the length of this peptide portion is determinant for the proper insertion, stability and efficient transport activity of the protein (Weinglass and Kaback, 2000; Monden *et al*., 2001). The function of the central loop in the regulation of transport activity has also been analyzed for sucrose transporters. It seems that in these peptides the intact central loop is not required for membrane insertion, plasma membrane

targeting, and transport (Reinders *et al*., 2002b), while it has been attributed a regulative role in the substrate affinity to the N-terminal portion of protein. In fact, when the N-terminus and central cytoplasmic loops of the low affinity transporter AtSUT2 were individually exchanged with the respective domains in the high affinity transporter StSUT1, it was observed that only the N-terminal is relevant for substrate affinity (Schulz *et al*., 2000). Moreover, it has been observed that a histidine at the position 65, localized in the first external loop, has an important role in substrate binding. In fact, the modification of this amino acid by site-directed mutagenesis has increased protein transport activity (Lu and Bush, 1998). The His-65 is a conserved amino acid that has been reported in all SUT proteins (Lalonde *et al*., 2004). Topological analysis with two site-specific antibodies against SUT also demonstrated that both C- and N-termini are localized on the cytoplasmic side of the membrane (Stolz *et al*., 1999), while a study of protein sequence comparison has identified two conserved domains located in the second and seventh cytosolic loops that are homologous to the [RK]X2-3[RK] motif found in a bacterial $H^+$-coupled sugar transporter, supporting the hypothesis that the structure of SUT protein arose from an ancestral gene duplication event (Ward *et al*., 1998).

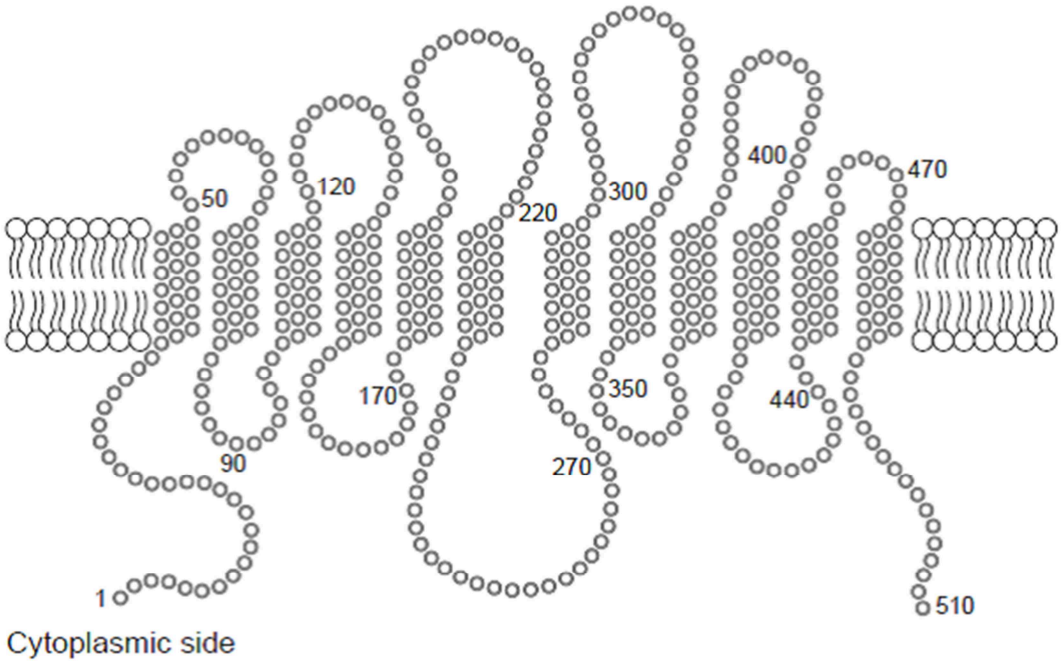


**Figure 7. Topological model of a sucrose transporter (Williams *et al.*, 2000)**

## 1.10. Sucrose transporters in dicotyledonous species

The classification of SUT proteins into different subfamily reflects either an amino acids sequence homology, and similar substrate affinity and function throughout phloem pathway (Kühn, 2003).

The multiple SUT genes found in higher plants exhibit distinct expression patterns, depending on tissue, development and plant species. They are expressed in a range of plant tissues that includes both source and sink organs, and hence play different function within phloem transport system (Kühn and Grof, 2010). As described above, sucrose transporters of dicotyledonous species fall into three distinct groups: SUT1, SUT2 and SUT4 subfamilies (Kühn and Grof, 2010).

### 1.10.1. SUT1 subfamily

Members of the SUT1 subfamily are classified as high affinity/low capacity sucrose transporters. When expressed in heterologous system, they exhibit a $K_m$ values in the order of about 0.05 mM, as AtSUC9 (Sivitz *et al*., 2007), to about 1.5 mM, for example AtSUC2 (Chandran *et al*., 2003). Being this group composed of members from only dicotyledonous plants, it has been suggested that it appeared after the divergence between monocotyledons and dicotyledons (Shiratake, 2007). In several plant species, such as Arabidopsis, common plantain, grapevine, rape and tobacco, gene duplications seem to have increased the number of genes belonging to this clade (Sauer, 2007).

Proteins of this group can transport several kinds of glucosides, some of which are secondary metabolites found in plants (Sivitz *et al*., 2007). For instance, SoSUT1 and StSUT1 are able to transport, beside sucrose, maltose with Km values of 5 mM and 10 mM, respectively (Riesmeier *et al*., 1992, 1993). AtSUC9 has low substrate specificity and can transport a wide range of glucosides, including helicin, salicin, arbutin, maltose, fraxin, esculin, turanose, and *α*-methyl-d-glucose (Sivitz *et al*., 2007). The Arabidopsis AtSUC5 as well as PmSUC2, others members of SUT1 subfamily, can also take up a structurally different compound, the biotin (vitamin H), when expressed in a biotin uptake-deficient yeast mutant (Ludwig *et al*., 2000).

It has been shown that SUT1 proteins play different role in the phloem transport pathway. Some members seem to be essential for sucrose export from leaves. In potato and tobacco, the reduction of SUT1 transcript abundance through antisense inhibition has led to an accumulation of carbohydrate such as glucose, fructose and sucrose in leaves, a decrease in plant growth, and, in the case of potato, a reduction in tuber yield. Taken together these results indicate an interruption of phloem loading, and hence attribute an essential function for SUT1 in sucrose uptake into phloem cells in source organs (Kühn *et al*., 1996; Lemoine *et al*., 1996; Bürkle *et al*., 1998). A similar function in phloem loading has also been attributed to AtSUC2. In fact, insertional mutants are strongly retarded in growth and development, and are unable to produce seeds (Sauer and Stolz, 1994).

Numerous homologs of SUT1 subfamily have also been detected in sink tissues indicating their putative role in sucrose partitioning also in this organs (Sauer, 2007). Moreover, the expression of GUS genes under the control of *LeSUT1* promoter in transgenic potato plants has been detected in sink tubers and sprouting source tubers, suggesting a role for SUT1 in non-photosynthetic source organs (Kühn *et al*., 2003). In addition, the specific sink-antisense inhibition of *StSUT1* using the tuber-specific patatin promoter, a gene which is primarily expressed in the phloem, led to a reduction of fresh weight accumulation during early stage of tuber development, suggesting a putative role of sucrose transporters on phloem transport in the sink organs (Kühn *et al*., 2003). Gene expression of *NtSUT3* and *AtSUC1* has been observed in pollen sink tissue. It has been attributed a role in the nutrition during pollen tube growth (Lemoine *et al*., 1999; Stadler *et al*., 1999), while mRNA of *AtSUC9* has been found in flowers. Plants containing mutation in *AtSUC9* gene have early flowering phenotype under short-day condition (Sivitz *et al,* 2007). Transcripts of SUT1 members have also been detected in fruit sink. For example, *VvSUT1* gene expression has been measured by quantitative PCR in all stages of fruit development, showing an accumulation at the onset of ripening (Ageorges *et al*., 2000) and a function in the load of sucrose into parenchyma cells from the apoplast has been assigned to this transporter (Manning *et al*., 2001).

### 1.10.2. SUT2 subfamily

Proteins of the second clade are named SUC3/SUT2-type transporters, based on the names of the first characterized transporters, AtSUC3 and LeSUT2. They are found in both monocotyledons and dicotyledons. In contrast to clade 1, no gene duplication events seem to have occurred within this clade (Sauer, 2007). SUT2 proteins are characterized by a different protein structure compared to all other SUT family members. In fact, they contain a more extended N-terminal, and an elongated central loop of approximately 20 and 60 amino acids, respectively, and the C-terminal is slightly shorter than SUT proteins belonging to clade 1 (Sauer, 2007). They have an additional feature, i.e. two highly conserved boxes CCB1 and CCB2 in the central loop, a low codon bias that cause a low translational efficiency and usually low expression levels (Barker *et al*., 2000). Moreover, for some members, symporter activity has not been detected; for example LeSUT2 and StSUT2 are unable to transport sucrose in heterologous system (Barker *et al*., 2000). This feature and the high degree of sequence similarity with SNF3 and RGT2 glucose sensors in yeast (Ozcan *et al*., 1998) allowed to hypothesize that LeSUT2 and StSUT2 may play a role in the perception of sucrose concentration as sucrose sensors (Barker *et al*., 2000). This hypothesis is still debated because there are some

contradictory evidences. For example, orthologs from other species, such as AtSUC3 and PmSUC3, have shown transport activity with a low affinity (Meyer *et al*., 2000; Barth *et al*., 2003). Moreover, the phenotype of a knockout mutant of Arabidopsis *Atsuc3*, the unique member of SUT2 clade in this species, has no noticeable differences compared to wild type.

Transcript encoding SUT2 sucrose transporters are generally found at low levels mainly in sink organs. For example, *LeSUT2* shows higher expression in sink leaves and stem then source leaves (Barker *et al*., 2000) and a greater expression in mature fruit compared to immature organ (Hackel *et al*., 2006). Moreover, the antisense inhibition of *LeSUT2* leads no growth alteration in transgenic plant, but a significantly reduction of fruit yield compared to the wild type, mainly due to a reduction in fruit size, rather than in the number or reproductive organ. Because fruits of antisense plants were sterile or contained only few seeds, a role in pollen development and pollen tube growth has been assigned to LeSUT2 (Hackel *et al*., 2006).

### 1.10.3. SUT4 subfamily

This clade, as SUT2 subfamily, includes monocot and dicot members. SUT4 proteins have only 47% similarity to SUT1 members (Weise *et al*., 2000). Like SUT2 clade, no gene duplication has been identified in this group (Sauer, 2007). Heterologous expression in yeast revealed their ability to catalyze sucrose uptake with low affinity and high capacity (Weise *et al*., 2000; Reinders *et al*., 2008). For example, the measurement of sucrose uptake in yeast expressing AtSUT4 and StSUT4 has shown a Km value for both proteins in the range of 6 mM (Kühn, 2003). The protein structure presents some differences compared to the other SUTs, in particular it shows a smaller C-terminal, and a very short sequence between the transmembrane helices VII and VIII. For this reasons, they usually have the shortest peptide sequence compared to all other SUTs in a given plant species (Sauer, 2007). Moreover, they can transport different types of substrate than SUT1 subfamily (Reinders *et al*., 2008).

SUT4 proteins have been identified in different plant species such as Arabidopsis, rice, tomato, carrot, potato, grape berry, and barley (Weber *et al*., 1997; Shakya and Sturm, 1998; Weise *et al*., 2000; Weschke *et al*., 2000; Manning *et al*., 2001). Distinct members of SUT4 subfamily are expressed in a variety of tissues. The transcription of GUS genes controlled by *AtSUT4* promoter has been detected at high level in sink leaves, in the restricted area of minor vein in source leaves, and in flowers (pistil and anthers) (Weise *et al*., 2000), while transcripts of *LeSUT4* and *StSUT4* were detected in source and sink leaves, in the ovaries of flowers, but also in green tomato fruits

(Kühn, 2003). Moreover, cDNA were isolated from apple and pear fruits. The expression of *PbSUT1* has been detected in all stages of fruit development with accumulation of transcripts during the ripening process (Zhang *et al*., 2013), while transcripts of *MdSUT1* were detected in a variety of tissues, such as leaves, shoots, flowers and fruits. MdSUT1 protein has been localized in the plasma membrane of both SE/CC complex and storage parenchyma cells (Peng *et al*., 2011). The analysis of spatiotemporal expression of *CsSUT4* revealed a higher amount of transcripts in mesocarp tissue enclosing the phloem than that in source leaves, sink leaves and stems (Hu *et al*., 2012)

The physiological role of SUT4 subfamily members is still debated. In fact, transgenic plant expressing SUT4 genes at lower level exhibited distinct phenotype in different plant species. In some case it has led no alteration of phenotype such as in *Arabidopsis*, whereas in other species opposite effects were observed. For example, increase or decrease of sucrose export from leaves, in potato and poplar, respectively (Chincinska *et al*., 2008; Payyavula *et al*., 2011).

# 2. CHAPTER 1: Mechanisms of sugar accumulation in peach mesocarp

## 2.1. Introduction

### 2.1.1. Sugar content in fruits

The carbohydrates content, generally determined by measurement of soluble solids content (SSC), significantly influences fruit taste and contributes to define quality of fleshy fruit. The main sugars or sugar alcohols encountered in fruits are sucrose, fructose, glucose and sorbitol. The composition of total sugars depends on several factors, including plant species, genotype variety, development stage or environment conditions (Shaw, 1988; Davies and Hobson, 1981; Wang and Camp, 2000). For example, in mature tomato (*Lycopersicon esculentum*) fructose and glucose are the predominant sugars with nearly equal amounts, while sucrose is present in trace quantities (Islam *et al*., 1996). A genetic control of total sugar concentrations, instead, is observed in melon. In fact, analysis of carbohydrate composition in different genotypes has revealed some differences in the level of total sugar accumulated with an association between high levels of total carbohydrates content and high levels of sucrose (Stepansky *et al*., 1999). This diversity in composition and concentration of stored sugars can be observed also within the same plant family. An example is the family of Rosaceae that includes the major temperate fruit crops, such as apple (*Malus domestica*), pear (*Pyrus communis*), almond (*Prunus dulcis*), cherry (*Prunus* spp.), peach (*Prunus persica* L.Batsch), plum (*Prunus* spp.) and strawberry (*Fragaria x ananassa*) (Potter *et al*., 2007). Although they belong to the same family, the sugars content and the proportion of the four major carbohydrates at fruit maturity greatly depend on the species and may be influenced by the variety. For instance, apple fruits accumulate mainly fructose and sucrose in the later phase of development (Li *et al*., 2012), strawberry fruits store glucose, fructose and sucrose, and therefore all three types of carbohydrates are important quality-determining factors in this species (Park, 2006), while sour cherry is characterized by a substantial sorbitol accumulation (Gao *et al*., 2003).

### 2.1.2. Carbohydrates content in mesocarp of *Prunus persica*

The carbohydrate content has been extensively analyzed in the mesocarp of peach fruit. Generally, the average values of soluble solids content obtained in commercial cultivars range between 9% and 15%, but in some cases it reaches up to 20% or more (Byrne *et al*., 1991; Crisosto *et al*., 1998). When individually analyzed, soluble sugars show different accumulation levels throughout fruit growth. In detail, glucose and fructose are present in nearly equal amounts during the early stage of development, whereas sucrose accumulates and becomes the predominant sugar in mature fruit, ranging from 45 to above 80% of total sugars. Sorbitol levels remain low throughout development (Moriguchi *et al*., 1990; Vizzotto *et al*., 1996; Lo Bianco *et al*., 1999; Morandi *et al*., 2008). Other alcohol-soluble sugars may be detected, but in low or barely amounts, such as inositol, mannose, xylitol and xylose (Layne and Bassi, 2008). Distinct cultivars differ in the proportion of soluble sugars stored, and this may influence the fruit quality. Peaches considered of "high-quality" contain large amounts of fructose, the sweetest sugar, and lower quantities of glucose and sorbitol than "low quality" cultivar (Robertson and Meredith, 1988).

### 2.1.3. Accumulation of sugars in fruit

The quantity of carbohydrates stored in mesocarp tissue depends on several factors that constitute different points of regulation along phloem pathway and are: photosynthesis, synthesis of translocation sugars, phloem loading, sugar translocation, phloem unloading, post-phloem transport, metabolic conversion and compartmentation (Figure 8).

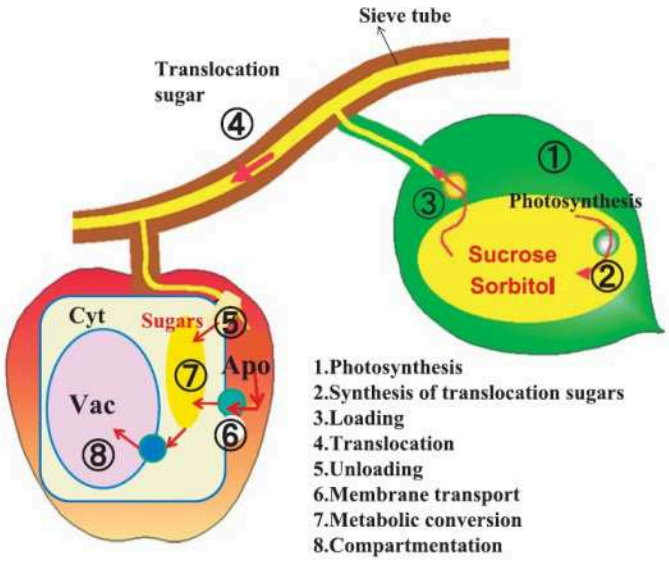


**Figure 8. Physiological steps that regulate photoassimilates movement from source leaves to sink fruits (from Yamaki, 2010). Apo: apoplast; Cyt: cytoplasm; Vac: vacuole.**

Among these, the physiological steps that determine fruit sink strength are those involved in the movement of sugars from phloem into sink tissue, i.e. unloading, post-phloem transport, and compartmentation, and those that determine the metabolic conversion of imported sugars (Yamaki, 2010).

### 2.1.4. Metabolic conversion in fruits

The main non-reducing sugar transported through phloem sap is the sucrose, but other types of carbohydrates may be detected. For example, in Rosaceae species, that comprise several important fruit crops, sorbitol is also used as a form of carbon translocated. For this reason, proteins which have a close relationship with sucrose metabolism and sorbitol metabolism have been extensively investigated in several fruits, in order to identify the key enzymes responsible for sugar accumulation. The main enzymes involved in the metabolic conversion of sucrose are sucrose synthase (SUSY), sucrose phosphate synthase (SPS) and invertase (INV), whereas sorbitol oxidase (SOX) and sorbitol dehydrogenase (SDH) are closely connected with sorbitol conversion (Figure 9).

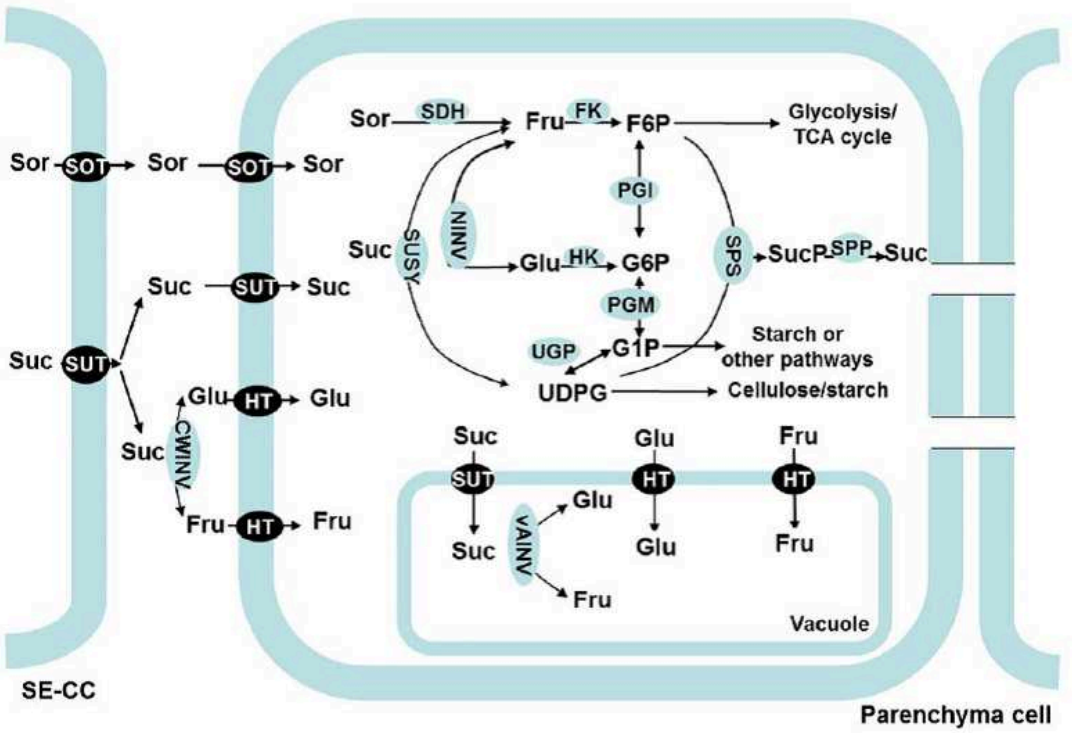


**Figure 9. Metabolic conversion and transport of sugars in apple fruits (from Li *et al.*, 2012).**

SUSY, a soluble enzyme located in the cytoplasm, catalyzes the reversible conversion of sucrose and UDP or ADP to UDP- or ADP-glucose and fructose (Baroja-Fernandez *et al.*, 2003). Generally, the reaction is considered to be directed towards breaking of sucrose, supplying cells of UDP-

glucose, product that is implicated in the synthesis of starch, callose and several cell wall polysaccharides (Asano *et al*., 2002; Subbaiah and Sachs 2001; Albrecht and Mustroph, 2003), but in some species the enzyme seems to be more involved in the sucrose synthesis. For example, analysis of SUSY activity, purified from peach fruit, showed a higher affinity for UDP-glucose compared to that from other plant species, suggesting a role in sucrose synthesis rather than in degradation (Moriguchi and Yamaki, 1988). A similar result was observed in pear fruit, in which two SUSY isozymes were isolated (a phosphorylated and an unphosphorylated protein). The latter form was detected to have a greater affinity for UDP-glucose and hence its activity is associated to sucrose synthesis, as SUSY from peach (Tanase and Yamaki, 2000; Tanase *et al*., 2002). SPS, another soluble enzyme localized in the cytoplasm, is responsible for the reversible conversion of UDP-glucose and fructose-6-phosphate (F-6-P) to UDP and sucrose-6-phosphate. However, in plant cells, its activity is essentially irreversible because the cytosolic concentration of sucrose-6-P is keep at low level by the activity of sucrose phosphatase. Thus, SPS is considered to be involved in sucrose synthesis (Huber and Huber, 1996). Several full-length cDNA were cloned from fruits of different plant species, such as citrus, melon, kiwi, pear (Komatsu *et al.*, 1999; Yu et al., 2007; Langenkämper *et al*., 2002; Itai and Tanahashi, 2008). The third key enzyme of sucrose metabolism, the INV, is responsible for irreversible sucrose hydrolysis to glucose and fructose. There are three widely accepted classifications of invertase, according to the subcellular localization, namely: neutral/alkaline or cytoplasmic invertase (CINV), apoplastic or cell wall invertase (CWINV, ionically bound to the cell wall matrix), and vacuolar invertase (VINV) (Winter and Huber, 2000).

Regarding sorbitol metabolism, the two main key enzymes that regulate sorbitol conversion are sorbitol oxidase (SOX) and sorbitol dehydrogenase (SDH). The cDNA encoding sorbitol dehydrogenase, that catalyses the conversion of sorbitol in fructose (Fayek and Wayne, 1979), has been isolated from several fruits, such as apple, pear and strawberry (Yamada *et al*., 1998; Oura *et al*., 2000; Duangsrisai *et al*., 2007). It has been shown that the protein generally has a lower Km value, about 100 mM, for sorbitol degradation as compared to sorbitol synthesis, consequently it easily converts sorbitol to fructose (Yamaki, 2010). The other key enzyme in sorbitol metabolism is the sorbitol oxidase that transforms sorbitol in glucose (Yamaki, 1980).

Other important enzymes are involved in sugar metabolism, such as fructokinase (FRK), that is involved in the phosphorylation of fructose and regulates the levels of fructose produced by the

conversion of sorbitol and by the hydrolysis of sucrose; ADP glucose pyrophosphorylase and starch synthase for starch synthesis; phosphorylase and amylase for starch breakdown.

The expression and activity of enzymes catalyzing the conversion of sugar imported into fruits show different regulation throughout development, depending on plant species.

In apple, both sorbitol and sucrose are imported into fruit sink. During the early stage of development the activity of SDH and FRK rapidly metabolize the sorbitol, while invertase and sucrose synthase convert sucrose into glucose and fructose. The products of the reactions are used as source of energy for cell division and fruit growth. In mature fruit, the activity of these enzymes decline allowing the accumulation of fructose into vacuole, while starch is stored in the insoluble form in plastids. During the last phases of fruit development, starch is degraded, and the activity of sucrose phosphate synthase contributes to the continued sucrose accumulation in the vacuole (Li *et al*., 2012).

In grape, where sucrose is the main form of carbon translocated, ripe berries store mainly glucose and fructose. The hexose accumulation starts eight weeks after flowering and continues until final development. It has been observed that the vacuolar invertase activity increases approximately when the concentration of soluble sugars begins to elevate, and remains constant throughout development, while the expression of two cytoplasmic invertases is high in immature fruit and greatly declines at the commencement of hexose accumulation (Davies and Robinson, 1996). Moreover, it has been observed that cultivars that differ in sucrose concentration show distinct levels of acid invertase activity (Wu *et al*., 2011). Therefore, the sugar accumulation in grape berry has been associated to the activity of different invertases.

Kiwifruit (*Actinidia* Lindl. spp.) accumulates a large amount of starch that is degraded into soluble sugars when the fruit starts to ripe. At cell division, the high concentration of glucose is associated with high neutral invertase activity, while the decrease of glucose concentration and neutral invertase activity occurs concurrently with transition to cell expansion and starch accumulation. The key enzyme identified to regulate starch metabolism and accumulation in the late period of development is the ADP-Glc pyrophosphorylase (AGPase). In fact, genotypes that accumulate high levels of the insoluble carbohydrate showed higher level of AGPase activity (Nardozza *et al*., 2013).

### 2.1.5. Carbohydrates metabolism in peach fruit

The first attempt to elucidate the mechanism of sugar accumulation in peach fruits was carried out by the analysis of activity of key enzymes involved in the main sugars conversion pathways. In

*Prunus persica*, like in other Rosaceae species, sucrose and sorbitol are both present in phloem sap as form of carbon translocated. For this reasons, enzymes regulating the metabolism of both sugars have been extensively investigated.

Several groups of researchers have measured the activity of the key enzymes in peach fruit carbohydrates metabolism during development, in order to elucidate the proteins responsible for the accumulation of sugars, mainly sucrose, and the major source of carbon that supply fruit growth, but different findings arose from different genotypes. The first effort was performed by Moriguchi *et al.* (1990) which, on the basis of an apparent relationship between sugar composition and relative enzyme activities, have hypothesized that glucose and fructose accumulation in young fruit of cultivar 'Hakuto' depends on acid invertase and sorbitol oxidase activities, while sucrose increase during the last phase of development is determined by the activity of sucrose synthase, operating in the synthesis direction. In fact, they did not detect any activity of other sorbitol-related enzymes, and a very low activity of SPS. They suggested that, in the early phase of fruit development, sorbitol is apparently converted to glucose by sorbitol oxidase, and sucrose hydrolyzed in glucose and fructose by invertase, while during the last phase of growth sucrose synthase produces sucrose from glucose and fructose. The authors have proposed that both sucrose and sorbitol could be used to support fruit growth, but they failed to explain how sorbitol contributes to growth in the last phase of development. Another work reported a similar result, but an increased in SPS activity in mature fruit was detected together with the raise of sucrose synthase activity, suggesting that both enzymes could participate to sucrose accumulation (Hubbard *et al*., 1991). In contrast, measurements of enzyme activity carried out on peach mesocarp of cultivar Redhaven throughout fruit growth showed higher levels of sucrose-hydrolyzing enzymes, namely insoluble acid invertase, soluble acid invertase, neutral invertase, and sucrose synthase, during the early stages of development, followed by a dramatic decrease accordingly with sucrose accumulation. Moreover, no significant increase in synthesis activity was detected. The authors suggested that sucrose enters the cell carbohydrate pool directly, and accumulates due to the disappearance of hydrolytic enzyme activities (Vizzotto *et al*., 1996). Another research group analyzed enzyme activity on vegetative and reproductive sinks of 'Encore' peach fruit focusing on the function of sorbitol as a carbohydrate source for supplying fruit growth. In contrast to Moriguchi *et al.* (1990), they detected no sorbitol oxidase activity during any stage of development, and only a small amount of sorbitol dehydrogenase during the final phases of fruit growth (Lo Bianco *et al*., 1999). The extension of the analysis to vegetative sinks lead the

authors to hypothesize that in peach tree sucrose is the major carbon form used for fruit growth, whereas sorbitol has a predominant role in vegetative growth (Lo Bianco *et al*., 1999).

### 2.1.6. Phloem unloading in fruit

The pathway of photosynthates release has been investigated over the past 20 years (Turgeon and Wolf, 2009) in several sink organs such as root apices, developing leaves, tubers, maternal tissues of seeds and fruits (Imlau *et al*., 1999; Haupt *et al*., 2001; Viola *et al*., 2001; Ruan *et al*., 2001). In fruits, the pattern of phloem unloading has been elucidated in a small number of plant species. These few investigation have highlighted that the unloading route can be interchanged between symplasmic and apoplasmic mechanism depending on genotype and development stage.

In apple fruit (*Malus domestica*), as also in cucumber (*Cucumis sativus* L.), several evidences have suggested an extensive apoplasmic phloem unloading pathway throughout sink development (Zhang *et al*., 2004; Hu *et al*., 2011). In both species, cytological analysis of phloem cells and physiological studies of 5(6)-Carboxyfluorescein (CF) movement in samples collected at different growth stages revealed that phloem strands are symplasmically isolated from parenchyma cells throughout development. In fact, plasmodesmata are quite absent or present in a very low quantity. Moreover, the fluorescent phloem tracer CF remained confined to the phloem strand. In apple, the content of total soluble sugars in phloem, cytosol and apoplasmic space confirms the hypothesis of an apoplasmic phloem unloading step, because it has been found being 100 mM, 930 mM, and 400 mM, respectively (Yamaki and Ino, 1992; Beruter *et al*., 1997). These concentrations rule out the hypothesis of an unloading driven by passive diffusion. Moreover, a putative monosaccharide transporter, localized to the plasma membrane of sieve elements and parenchyma cells, may be involved in the movement of sorbitol across the membranes. In fact, its expression increases during development (Zhang *et al*., 2004).

A similar pattern has been observed in jujube fruit (*Zizyphus jujuba*), but in this plant system a predominantly apoplasmic unloading mechanism is interrupted by a transient symplasmic phase at the middle stage of growth (Nie *et al*. 2010). In fact, during this period numerous plasmodesmata have been observed in the interface between SE/CC complex and adjacent cells or between phloem parenchyma and flesh parenchyma cells, while in immature and mature fruits, they were almost totally absent or blocked by electron-opaque globules. The presence of an intermediate phase characterized by a symplasmic route of phloem unloading was confirmed by the observation that CF was apparently released from the phloem into surrounding cells.

Furthermore, activity of cell wall invertase, responsible for the hydrolysis of sucrose in the apoplast was detected during early and late stages, when the pathway of unloading is supposed to be apoplasmic.

The shift of unloading routes in response to sink development has also been observed in tomato (*Lycopersicon esculentum*) and grape (*Vitis vinifera*) fruit. In both cases, a symplasmic mechanism occurs at early stages, while an apoplasmic pathway operates at the late stage of development (Ruan and Patrick, 1995; Zhang *et al*., 2006). In grape berry, the turning point has been observed to be at or just before the onset of ripening, when the fruit begins to store soluble sugars. Structural investigation showed that SE/CC complex and adjacent cells are symplasmically connected through functional channels, while a small quantity of plasmodesmata present an electron-opaque globule-obstruction under electron microscope in mature fruit. The CF and *fluorescent tagged viral movement protein* apparently remained confined to phloem cells only during sugar accumulation phase, when a cell wall acid invertase increases its activity. In addition, a high sugar level has been measured in the apoplast indicating a release of carbohydrates through the activity of plasma membrane proteins (Brown and Coombe, 1985; Patrick, 1997).

Finally, in walnut fruit (*Juglans regia*) distinct unloading patterns have been found in two types of tissues: symplasmic in seed, and apoplasmic in fleshy pericarp (Wu *et al*. 2004). The fluorescent dye, in fact, remained confined to the phloem strands of the sepal bundles in the fleshy pericarp, but released from the phloem strands of the minor ventral carpellary bundles into the surrounding parenchyma cells in the seed pericarp.

As a rule, the symplasmic pathway has been associated with lower resistance and greater transport capacity as compared with apoplasmic unloading (Patrick, 1997; Patrick and Offler, 1996).

### 2.1.7. Sugar transport in mesocarp tissue of peach fruit

The phloem unloading in peach fruit has not still investigated. However, the uptake kinetics of $^{14}$C-glucose and $^{14}$C-sucrose have been determined in peach mesocarp at three different growth stages, corresponding to a fruit at early, middle and late stage of development (Vizzotto *et al*., 1996). Mesocarp of young peaches are actively able to uptake sugars “in vitro”, with a saturable component sensitive to the uncoupler carbonylcyanide m-chlorophenylhydrazone (CCCP). This suggests the existence of a carrier-mediated transport, while the pattern of assimilate acquisition varies in mature fruit, and that the kinetic of assimilate acquisition in sink organ of *Prunus persica*

may involve an apoplasmic step and is developmental regulated. Together with these findings, different levels of extracellular invertase activity, responsible for the hydrolysis of sucrose into glucose and fructose in the apoplast, have been detected in young fruits.

The distribution of sugars among different compartments (free space, cytoplasm and vacuole) of peach mesocarp at two different growth stages has been determined, and it has been found that in young fruit sugars are mainly localized in the cytoplasm, while in mature fruit and predominantly located in vacuole. Moreover, the analysis of membrane permeability for different soluble sugar (sucrose, glucose, fructose and sorbitol) revealed that the velocity constants of carbohydrates across the plasma membrane were lower in mature fruit compared to younger fruit, indicating that sugars could pass through the plasma membrane more easily in immature fruit (Jiang *et al*., 2013).

## 2.2. Methods

### 2.2.1. Plant material

Experiments were conducted on fruits harvested from peach plants of cultivar Redhaven (*Prunus persica* (L.) Batsch) cultivated in the Experimental Farm "A. Servadei" of Udine University in north-eastern Italy (46.01N, 13.13E). Trees received ordinary horticultural care. Fruit growth was monitored weekly from about 30 day after full bloom (dafb) until harvest by measuring the transverse diameter and fresh weight of a pool of fruits collected from ten different plants. Several pool of mesocarp tissues were frozen in liquid nitrogen and stored at -80°C for subsequent analysis and flesh pieces of about 4 $mm^2$ in size, representative of different development stages, were produced for following embedding procedures (see 2.2.2).

### 2.2.2. Fixation, dehydration and embedding of tissues

In order to maintain the morphology and the integrity of fruit tissues, mesocarp samples were fixed and embedded as described by Christensen *et al*. (1998), with some modifications. All the steps were performed in slow agitation. Fruits were collected at different growth stages and cutted in small pieces of about 4 $mm^2$, hence immediately incubated in a fixative (45% ethanol, 5% formalin [40% paraformaldehyde], 5% acetic acid) for two days at 4°C. Subsequently, tissues were dehydrated through a series of washing in ethanol solutions at growing concentration: 50%, 70%, 96% and 100%. Each step was done twice for 1 hour, except the last stage that was performed overnight. The following day, they were firstly transferred in a solution containing 50% ethanol and 50% limonene (Roti®-Histol, Roth) and then two times in limonene alone for 2 h each. Hence, half solution was replaced with liquid paraffin (paraplast-plus, McCormick) and samples incubated at 60°C for 2 h. Afterwards, the limonene/paraffin solution was discarded, substituted with paraffin alone and samples maintained at 60 °C for other 2 h. Finally they were transferred in new paraffin and incubated overnight at 60°C. Inclusion blocks were prepared by means of HistoStar Embedding Workstation from Thermo Scientific.

### 2.2.3. Carbohydrate determination

The carbohydrates content (sucrose, glucose and fructose) in mesocarp tissues sampled weekly throughout development was determined by a spectrophotometric method combined with enzyme-catalyzed reactions. Firstly, sugars were extracted from 1 g of tissue as described in Nonis

*et al*. (2008). Material was incubated in different ethanol solutions at decreasing concentration (once in 5 ml of 100% ethanol, twice in 7.5 ml of 60% ethanol, twice in 7.5 ml of 20% ethanol and twice in 7.5 ml deionized water). Each step was performed for 10 min at 60°C, with the exception of the first step that was done for 10 min in boiling water. Each incubation time was followed by centrifugation at 4500 g for 3 min at room temperature, after which the supernatant containing sugars was recovered in new tubes and stored at -20°C for subsequent analysis.

Sugars quantification was carried out transforming carbohydrates through enzymatic reactions, and measuring photometrically the content of NADPH, one of the products of the reactions, that absorbes at 340 nm. The reactions and the relative enzymes employed are summarized in Figure 10. Four different enzymes were used: invertase that hydrolyzes sucrose into glucose and fructose, hexokinase (HK) that phosphorylates glucose or fructose in the presence of ATP to produce glucose-6-phosphate (G6P) or fructose-6-phosphate (F6P), phosphoglucose isomerase (PGI) that converts F6P to G6P and finally glucose-6-phosphate dehydrogenase (G6P-DH) that in the presence of NADP catalyzes the G6P oxidation in gluconate-6-phosphate with the formation of NADPH. The conversion of glucose, fructose and sucrose in gluconate-6-phosphate and NADPH allows to measure the reduced form of NADP that is stoichiometric to the amount of the three carbohydrates presents in the sample. In detail, glucose was phosphorylated to G6P that was hence converted to gluoconate-6-P and NADPH; sucrose was firstly hydrolysed in fructose and glucose and the latter was converter as above described. Finally, fructose is phosphorylated to F6P that is converted to gluconato-6-P and NADPH by G6P-DH.

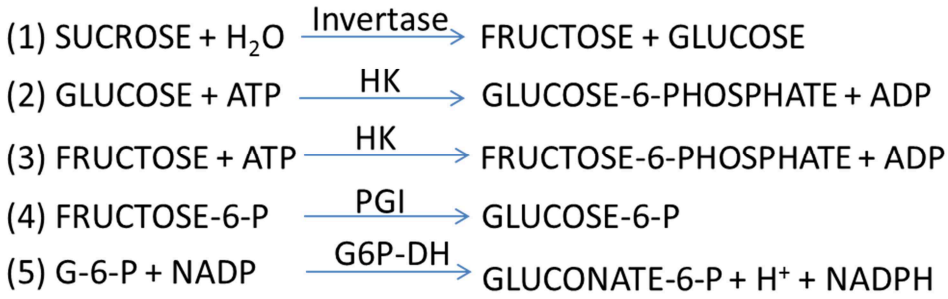


**Figure 10. Enzymatic reactions used to quantify carbohydrates content in peach mesocarp.**

All the reactions were done in 96-well microtiter plates and the absorbance measured through microplate reader TECAN Genios. For each samples, sugars content was measured in triplicate in order to calculate the standard deviation.

Glucose concentration was determined before and after hydrolysis of sucrose. Each sample (5 µl) was incubated in 10 µl citrate buffer (0.16 M Trisodium citrate dehydrate, 0.16 M Citric acid monohydrate, pH 4.6) in the presence (for sucrose quantification) or absence (for glucose quantification) of 1 µl invertase (Sigma), for 30 min at 37°C, after which 50 µl Triethanolamine buffer (0.75 M Triethanolamine hydrochloride, 0.01 M $MgSO_4 \cdot 7H_2O$, pH 7.6), 80 µl deionized $H_2O$, 5 µl NADP 11.5 mM and 5 µl ATP 81 mM were added. After 3 min a first reading at 340 nm was made. Then 1 µl HK/G6P-DH (Sigma) was added and the absorbance of NADPH measured 15 min later for several times until lecture stabilization. Glucose concentration was obtained simply measuring samples not treated with invertase, while sucrose amount was calculated by subtracting the absorbance of sample incubated and not with invertase. To quantified fructose, absorbance was measured after adding of 1 µl PGI to the sample without invertase. The sugars levels were calculated by calibration curve prepared from glucose, fructose and sucrose standard solutions (1 mM, 2.5 mM, 5mM and 10 mM).

### 2.2.4. Methylene blue staining

Mesocarp sections were stained with methylene blue solution, a dye which binds cellulose, and observed at optical microscope to study the distribution of vascular strands in the whole fruit. Tissue sections of 10 µm in thickness were cut by means of the microtome Leitz 1512, put on water, then retrieved and placed on slices. Subsequently, they were outstretched through a step on warm water and dried at 37°C for 1 h. Before staining with methylene blue, sections were de-waxed by two steps in limonene (Roti®-Histol, Roth) 10 min each and rehydrate by transfers in ethanol solutions at decreasing concentration (100%, 95%, 70%, 50% and 30%) terminated with two steps in deionized water. A drop of 0.1% aqueous methylene blue was placed on each section and left for 5 min. The dye was replaced with deionized water and samples were re-hydrated through steps in ethanol solutions at increasing concentration (30%, 50%, 70% 95% and 100%). Finally a drop of mounting (CC/Mount™ from Sigma) and a cover-slide were placed on the sample.

### 2.2.5. Carboxyfluorescein

The fluorescent dye, carboxyfluorescein was used to study phloem transport through the peach mesocarp. Analysis of carboxyfluorescein transport was carried out on peach fruits collected at different growth stages, at about 25 dafb, 75 dafb and 110 dafb, corresponding to fruit at early,

middle and late period of development, respectively, in order to clarified the unloading mechanism adopted by peach sink organ.

The 6(5) carboxyfluorescein (CF) is a compound often used as a marker to analyze the mechanism of phloem unloading in sink tissue. Due to its impermeability to the membrane and its fluorescence, it allows to figure out how the photoassimilates move from phloem to parenchyma cells. The tissue is firstly supplied with a solution containing 6(5) carboxyfluorescein diacetate (CFDA), the membrane-permeable and non-fluorescent precursor of CF that is degraded to carboxyfluorescein by intracellular esterases as soon as it is loaded into cells becoming fluorescent and membrane-impermeable. Its movement from phloem to sink cells can occur only through the plasmodesmata, whereby solely when there is symplasmic continuity between cells, given by the presence of functional channels (Roberts *et al*., 1997; Viola *et al*., 2001).

A water solution containing 5(6)-Carboxyfluorescein diacetate (Sigma) 1 mg/ml was prepared from a stock solution in acetone. For each growing phase, a branch carrying some leaves and one fruit was fed with CFDA aqueous solution for at least 5 h. The treated fruits were subsequently hand sectioned and examined directly by inverted fluorescence microscope (Axiovert35, Zeiss). The optimal λ for CF excitation and emission are 492 nm and 517 nm, respectively. Pictures were taken by Nikon camera connected to the microscope.

### 2.2.6. Sequence analysis

Genes encoding sucrose transporters from *P. persica* were identified by whole genome BLAST search on Phytozome web site (http://www.phytozome.net/search.php?method=Org_Ppersica) using SUT proteins from *A. thaliana* as a query. Proteins belonging to the same families from different plant species were selected to carry out a multiple alignment and used to create a sequence similarity UPGMA-tree by means of MEGA version 5 (Tamura *et al*., 2011).

The Compute PI/MW web tool of ExPASy (http://web.expasy.org/compute_pi/), from Bioinformatics Resource Portal of SIB (Swiss Institute of Bioinformatics) was employed to calculate the putative molecular weight of the identified proteins, while the putative amino acids sequences were analysed by taking advantage of the TMHMM Server v. 2.0 web-based service (http://www.cbs.dtu.dk/services/TMHMM-2.0/) of the Center for Biological Sequence Analysis (Technical University of Denmark) to calculate the probability of a region to form a transmembrane domain (from 0 to 1).

### 2.2.7. RNA isolation and cDNA synthesis for Real-Time PCR analysis

Total RNA was extracted according to the method described by Nonis *et al*., (2007) from pooled samples of peach mesocarp stored at -80°C, harvested at different development stages during growing season anno from 10 different plants in order to randomize biological variations. For each sample, 2.5 g were weighed and transferred in a tube containing 7.5 ml lysis buffer (150 mM Trizma base, 50 mM EDTA disodium salt, 4% Sodium dodecyl sulfate, pH 7.5) and 0.3 g polyvinylpolypyrrolidone (PVPP) preheated at 65°C. Afterwards, 75 µl of β-mercaptoethanol, 750 µl K-Acetate 5M, 2 ml ethanol 100% and 10 ml chloroform:isoamyl alcohol (24:1) were added. Tubes were centrifuged at 6000 g for 20 min at 4°C, after which the aqueous phase was recovered and transferred in new tubes together with 10 ml phenol chloroform:isoamyl alcohol (25:24:1). A second centrifugation was performed at 3700 g for 10 min at 20°C. The aqueous phase was again recuperated and transferred in new tubes. A second treatment with 10 ml chloroform:isoamyl alcohol (24:1) was made, followed by a centrifugation at 3500 g for 10 min at 4°C. The aqueous phase was recovered; 0.3 volumes of LiCl 12 M were mixed and samples were incubated overnight at -20°C. During the second day, samples were centrifuged at 16500 g for 15 min at 4°C. Then, supernatant was discarded and pellet washed with 10 ml LiCl 3 M. After a centrifugation at 16500 g for 15 min at 4°C, supernatant was again discarded and pellet air dried. Then, RNA was resuspended in 2.82 ml $H_2O$ DMDC (Dimethyl dicarbonate), and after the addition of 180 µl K-acetate 5M and 6 ml ethanol 100%, samples were vortexed and incubated for 1 h at -80°C. Subsequently, tubes were centrifuged at 19000 g for 12 min at 4°C, supernatant was discarded and pellet washed with 5 ml ethanol 75%. The last centrifugation was performed at 19000 g for 12 min at 4°C and afterwards supernatant discarded. Finally, pellet was air dried, resuspended in 50-200 µl $H_2O$ DMDC and transferred in new 2 ml tubes.

An aliquot of RNA was used to quantify samples by NanoDrop 1000 (Thermo Scientific). The integrity was checked on 1% agarose gel. In order to remove contamination by genomic DNA, a treatment with DNase (Promega) was performed as follows: 20 µg RNA, 10 µl 10X Reaction Buffer, 1 µl Rnasin Ribonuclease Inhibitor (Promega), 10 µl DNase 1U/µl and $H_2O$ DMDC to 100 µl. The reactions were incubated at 37°C for 30 min, after which RNA was purified and concentrated with Rneasy MinElute CleanUp Kit (Qiagen) according to the manufacturer's instructions. Then, samples were quantified by NanoDrop 1000 (Thermo Scientific), checked on 1% agarose gel and stored at -80°C for subsequent analysis.

The synthesis of complementary DNA was performed through SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen™). The following components were combined: 2.5 µg RNA, 4 µl 5X VILO™ Reaction mix, 10X SuperScript® Enzyme Mix, 1 µl oligo dT (10 µM) and $H_2O$ DMDC to 20 µl. The reactions were incubated in the Thermal Cycler 2720 (Applied Biosystems) according to this scheme: 10 min at 25°C, 120 min at 42°C and 5 min at 85°C. Hence, samples were stored at -20°C.

### 2.2.8. Quantification of gene expression by Real-Time PCR

Experiments of *PpSUT* transcript quantification were performed by Real-Time PCR with Opticon 2 (MJ Research) detection system. Specific primers were designed by primer3 web tool (http://frodo.wi.mit.edu/primer3/), tested and selected in order to have an efficiency of amplification approximately equal or close to 100%. In fact, the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), that assumes an equivalent amplification efficiency of target and reference genes, was employed to calculate the relative changes in transcript levels. All primers are listed in Table 3. Their efficiency was tested through the running of a standard curve, measuring the amplification of a 2X cDNA serial dilution. Moreover, their specificity was checked by melting curve analysis. All quantifications were carried out in triplicate in order to calculate standard deviation due to technical errors, with two different dilution of cDNA. The mean Ct values of target genes were normalized against the amount of reference gene, the ubiquitin-conjugating enzyme E2 (accession number BF717254) (Nonis *et al*., 2007) amplified at the same conditions. As a negative control, a reaction with water instead of cDNA was added. The following components were mixed: 9 µl 2.5X Real Master Mix SYBR ROX /20X SYBR Solution (5Primer), 0.4 µl forward primer (10 µM), 0.4 µl reverse primer (10 µM), 5.2 µl $H_2O$ DMDC and 5 µl cDNA. The thermal cycling was programmed as follows: one step at 50°C for 2 min, a first denaturation cycle at 95°C for 3 min followed by 41 cycles at 94°C for 15 sec, 56°C for 20 sec and 72°C for 30 sec, 1 cycle at 80°C for 1 sec and a final step at 72°C for 5 min.

**Table 3. Primers used to quantify gene expression by Real-Time PCR**

| gene name | primer forward | primer reverse |
|---|---|---|
| **ubiquitin-conjugating enzyme** | CCCACCTGATTACCCTTTCA | GGATCTGTCAGCAGTGAGCA |
| ***PpSUT1* (ppa004033)** | CTATGGCTCCCGCAATAAAG | CAGTATTCCCTTCGCTCTGG |
| ***PpSUT2* (ppa003041)** | AATACGGTGCAGGGACCAG | AACCATCTGTGCCAACTTCC |
| ***PpSUT4* (ppa004620)** | ATGTGACTCAGGGTCCTTGC | ACCAACCGCCATAAACAGAG |

### 2.2.9. Laser Microdissection Pressure Catapulting (LMPC)

Mesocarp tissue of about 5-6 fruits (cultivar Redhaven) collected at different growth stages were cut in approximately 2-3 mm wide and 4-5 mm long pieces in ice-cold ethanol : acetic acid (3:1; EAA), and fixed in fresh EAA overnight at 4°C. Before fixing, vacuum was applied three times for a few seconds and rapidly released. Fixed samples were dehydrated in glass vials at room temperature in a graded ethanol series [70% (v/v) ethanol for 60 min; 90% (v/v) ethanol for 60 min; 100% (v/v) ethanol for 60 min, twice; 100% (v/v) xylene for 60 min, twice]. Then, specimens were transferred into plastic cassettes and infiltrated with xylene: Paraplast Plus (1:1) (Mc Cormick Scientific, St Louis, MO, USA) at 59°C for 60 min. The mixture xylene : Paraplast was replaced with pure Paraplast three times at intervals of 60 min. Fixed specimen were embedded in Paraplast. Blocks were first cooled down to RT and then placed at 4°C for easy un-molding. The blocks were kept in plastic bags at 4°C. Ten-µm-thick slices were sectioned on a rotary microtome (Leica, Bensheim, Germany). Sections were stretched at 42°C for a few seconds on a drop of DEPC-water delivered directly on the PEN-covered glass slides (P.A.L.M. Microlaser Technologies, Carl Zeiss MicroImaging GmbH, Germany), and dried at 42°C for not more than 30 min. Sections were de-paraffinized two times for 5 min each in xylene and then air-dried. Phloem tissue and parenchyma cells were separately microdissected from the de-paraffinized sections with a P.A.L.M. Laser-Microbeam System (Carl Zeiss MicroImaging GmbH, Germany). Areas of at least 2 x $10^6$ $\mu m^2$ in total were cut and catapulted in 0.2 ml tubes with adhesive cap (P.A.L.M. Carl Zeiss).

### 2.2.10. RNA isolation and amplification from LMPC-captured cells

RNA from LMPC-captured cells was extracted using the Absolutely RNA Nanoprep Kit (Stratagene, Agilent Technologies, Inc., Santa Clara, CA, USA) with minor changes with respect to the manufacturer's instructions as already described in Santi *et al.* 2013. DNase-treated RNA was eluted in 14 µl of RNase-free water heated to 60°C.

T7 RNA polymerase-based RNA amplification was performed using the MessageAmp II aRNA Amplification Kit (Ambion, Life Technologies Co., Carlsbad, CA, USA) according to the manufacturer's instructions as already described in Santi *et al*., 2013. Nucleic acid quantity and integrity were evaluated using an RNA 6000 Pico Assay kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

### 2.2.11. Real-time RT-PCR from samples collected by LMPC

For gene expression analysis of LMPC-collected cells, 10 µl of amplified RNA (aRNA) was reverse-transcribed using random hexamers and the Superscript® III Reverse Transcriptase (Invitrogen, Life Technologies Co., Carlsbad, CA, USA) in a total volume of 20 µl. The cDNA synthesis reaction mixture was diluted 1:10 and 1:20, and used to quantify gene expression as described in 2.2.8..

### 2.2.12. In situ hybridization

Experiments of in situ hybridization were carried out to localize and detect *PpSUT* transcripts in morphologically preserved mesocarp sections at different stages of fruit development, hybridizing complementary RNA probes labeled with digoxigenin (DIG) to the sequences of interest. All the steps were performed using materials and solutions RNase free, to minimize the RNA degradation.

### 2.2.13. RNA probes synthesis

The DIG-RNA probes were generated by *in vitro* transcription through the cloning of a defined portion of *PpSUT* sequence into a dual promoter transcription vector, with the aim of synthesize two kinds of riboprobes, in sense (as a control for the specificity of the hybridization reaction) and antisense direction (complementary to the target mRNA). Firstly, for each gene, specific primers were designed by primer3 web tool (http://frodo.wi.mit.edu/primer3/) in order to amplify by PCR DNA fragments to clone into TA cloning dual promoter vector. The sequences of primers are listed in Table 4. The Taq DNA polymerase High Fidelity from Invitrogen™ was employed in the PCR

reaction because performs a high-fidelity amplification of nucleic acid templates and the majority of products amplified have 3-A' overhangs indispensable for the cloning with TA cloning system.

**Table 4. Primers used to amplify *PpSUT* sequence region selected as RNA probes.**

| gene name | primer forward | primer reverse |
|---|---|---|
| ***PpSUT1* (ppa004033)** | GTGACGACCCGAAGAGAATG | GACATGAACCCCAAAACGAC |
| ***PpSUT2* (ppa003041)** | TCGTGCTCTTCTGGCTGAT | TGCTGGTGGTAAATGCCTTA |
| ***PpSUT4* (ppa004620)** | GCTGATCTCACTGCAAAGGA | CCGAGTTCAACATCAGACCA |

**>*PpSUT1* probe**
**GTGACGACCCGAAGAGAATG**CGGACCGCCAACTCCCTGTTTGCGTTCTTCATGGCGGTCGGCAACGTCTTGGGGTA
CGCGGCCGGGGCCTACTCCCACCTCCACAAGATGTTCCCCTTCACCATAACCAAAGCATGCGACGTCTACTGCGCCA
ACCTCAAGAGCTGCTTCTTCCTTTCCATCACTCTCCTCCTCGTCCTCACCATCGTGGCCTTGACGTCGGTGAAGGAAA
CGACGCCGAATGACGGCGTCGTCGCGGAGGGGGAGATCGAGCCCCAGTCAACGACAGCTAAGTCCGTGCCGTTTT
TCGGTCAAATGATTGCGGCGTTCAGGGAGCTGCGGAGGCCAATGCTGGTGTTGCTTCTGGTGACGTGTCTCAATTG
GGTTGCATGGTTCCCGTTCTTGCTATTCGACACTGATTGGATGGGGCGGGAGGTGTACGGAGGCCAAGTTGGGAAG
GGGCGGTTGTACGATTTGGGGGTGAGGGCTGGTGCCCTTGGGCTGATGCTAAACGCC**GTCGTTTTGGGGTTCATGT
C**

**>*PpSUT2* probe**
**TCGTGCTCTTCTGGCTGAT**CTAGCAGGCCCTGAACAACGTAACACTGCAAATGCTGTGTTTTGCTCATGGATGGCCGT
TGGTAACATCCTAGGATTTTCTGCCGGTGCAAGTGGAAGTTGGCACAGATGGTTTCCTTTCTTGTTGAGTAGAGCTT
GCTGTGAAGCTTGTGGAAATCTTAAAGCAGCATTTCTTATTGCAGTGCTCTTTCTCACTTTGTGTACGCTTGTTACCAT
ATATTTTGCTGATGAGGTTCCACTCACTACGCATAAGACCAACCGTTTATCGGATGCTGCTCCTTTATTGGAGGATCCC
CAACAAAATGGCCTTGATCTTTCAAAATTAAAGCCTGATAAGCAAGTTATAGATAATGCAAATCAAAGCAGAACTGTG
AATGACTATGAAAGGGATATACATCTAAAGGAGGCCATCTCAAAAGTTGAAGAAGATAAGAATGGTGGTTTTAATGA
CGGACCTGGGGCCGTTTTAGTCAATCTATTGACCAGTT**TAAGGCATTTACCACCAGCA**

**>*PpSUT4* probe**
**GCTGATCTCACTGCAAAGGA**TTATCGAAGAACTCGAGTGGCAAATGCTTATTTCTCTCTGTTTATGGCGGTTGGTAAT
GTTCTTGGCTATGCAACTGGATCATTCAGTTACTTGTACAAGGTTTTTCCATTTTCAATTACCTCTGCGTGCAATATTAA
CTGTGCAAATCTCAAGTCTGCTTTCTTGGTCGACATTGCCTTCATTGTAATTACTACGTGTGTAAGCATATCAGCAGCTC
AGGAATTACCTCTGAGTTCAAGCAACAGTACTACACCCTTTTCTGAGGAAGGGCCAGGACAGTCGAGTCATGCTGA
AGAAGCTTTTCTCTGGGAGCTTTTTGGGACTTTTAGATATTTCTCAGGGTCTATATGGATAATCCTACTTGTTATTGCTC
TAAACTGGATCGGGTGGTTTCCATTTCTTCTCTTTGATACCGATTGGATGGGTCGAGAGATTTATGGTGGCAAGCCAA
ATGAAGGGGTAAATTATAGTACAGGTGTTAGAATGGGTGCTCT**TGGTCTGATGTTGAACTCGG**

**Figure 11. Probe sequence of *PpSUT1* (535bp), *PpSUT2* (526) and *PpSUT4* (535).**

The three probe sequences (Figure 11) were amplified from two different cDNA samples, produced from RNA isolated from seed and mesocarp, both prepared from a pool of samples collected at different growth stages.

The PCR reaction was composed of 22.5 µl PCR SuperMix High Fidelity, 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10 µM), 1.5 µl cDNA (10 ng/µl); the cycling program used was the

following: 1 cycle at 94°C for 30 sec, 35 cycles at: 94°C for 30 sec (denaturation), 58°C for 30 sec (annealing) and 68°C for 35 sec (extension), and finally 68°C for 5 min. The reactions were performed in the Thermal Cycler 2720 of Applied Biosystem®. The products were purified from gel using the Invisorb® Spin DNA Extraction Kit (Invitek, STRATEC Molecular) as described by the manual. The cleaned products were quantified by NanoDrop 1000 (Thermo Scientific) and checked on 1% agarose gel.

Hence, the fresh products were directly cloned into pCR®II dual promoter vector using TA cloning® kit Dual promoter from Invitrogen™, according to manufacturer's instructions. The pCR®II vector contains two antibiotics resistance (kanamycin and ampicillin) and the LacZα gene for the selection of the transformants. Firstly, the amount of PCR product was calculated with the following formula:

$$X\ ng\ PCR\ product = \frac{(Y\ bp\ PCR\ product)(50\ ng\ pCR^{®}II\ vector)}{(size\ in\ bp\ of\ the\ pCR^{®}II\ vector, \sim 3900)}$$

Then, the ligation reactions were prepared as follows: X µl fresh PCR product (to have two different ratio vector:insert, 1:1 and 1:3), 1µl 10X Ligation Buffer, 2 µl pCR®II vector (25 ng/µl), sterile $H_2O$ to 9 µl and 1 µl T4 DNA Ligase (4 units/µl); a negative control, prepared using sterile $H_2O$ instead of PCR product, was added. The reactions were incubated overnight at 14°C in Thermal Cycler 2720 (Applied BioSystem®).

Ligation reactions were used to transform chemically competent *E.coli* cells (TOP10 F' One Shot® cells from Invitrogen™). Briefly, 2 µl of each ligation reaction were added directly into a vial of competent *E.coli*. Cells were incubated 30 min on ice, then heated at 42°C for 30 sec and transferred immediately on ice. After added of 250 µl S.O.C. medium, vials were incubated at 37°C for 1 h at 225 rpm in a shaking incubator. Finally, 25 µl and 100 µl of each transformation were plated on LB agar plates containing 100 µg/ml ampicillin, 40 µl of 100 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) and 32 µl of 50 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Plates were incubated overnight at 37°C. The transformants were checked through a colony PCR. For each construct, 6 white colonies were picked, replicated in a new plate (containing 100 µg/ml ampicillin, 40 µl of 100 mM IPTG and 32 µl of 50 mg/ml X-Gal) and used as a template in a PCR with probe specific primers. The PCR mix and the thermal cycles were the same as described above for the amplification of cloned fragments. PCR products were checked on 1% agarose gel.

Subsequently, for each construct, one colony was chosen and grown in 5 ml of liquid LB containing 100 µg/ml ampicillin, overnight at 37°C. The following day, 600 µl of bacterial culture were added to 800 µl of glycerol 80%, mixed and stored at -80°C, while 4ml were used to purified the plasmid through the kit Wizard® Plus Minipreps DNA Purification System (Promega) according to the manufacturer's instructions. The purified plasmids were sequenced to check the orientation of the PCR product into the pCR®II vector, using two different primers, M13 forward (5'-TGTAAAACGACGGCCAGTG-3') and M13 reverse (5'-CAGGAAACAGCTATGAC-3'), located on either side of the insertion site. Each vector was linearized in order to stop the in vitro transcription after the PCR product and to avoid the insertion of the plasmid sequence into riboprobe. For each construct, two different reactions were performed with two different restriction enzymes, cutting upstream and downstream of the insertion site. The probe sequences were analyzed through the NEB cutter web tool (http://tools.neb.com/NEBcutter2/index.php) to choose a restriction enzyme that didn't cut inside the cloned sequence (Figure 13). BamHI was used to cut upstream construct carrying *PpSUT1* probe sequence, while KpnI to digest upstream plasmids containing *PpSUT2* and *PpSUT4* probe sequences. EcoRV was employed to restrict all three constructs downstream of the insertion site.

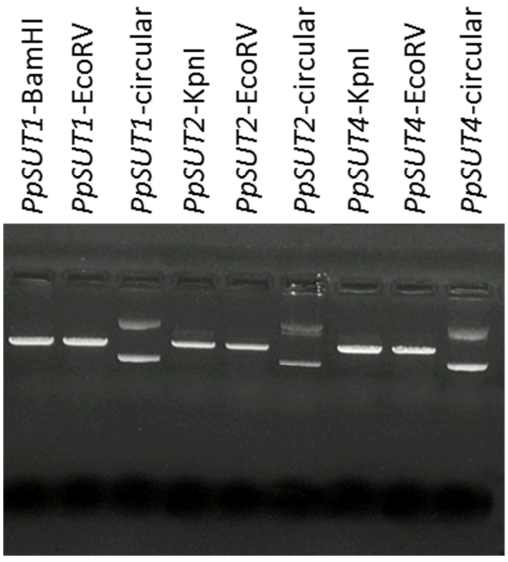


**Figure 12. Control of plasmid digestion on 1% agarose gel by the comparison between linearized and circular plasmids.**

The digestion reactions of plasmids, performed using restriction enzyme from Promega and composed of 2 µl RE 10X Buffer, 0.2 µl Acetylated BSA 10 µg/µl, 1 µg plasmid, 0.5 µl restriction enzyme (10 U/µl) and $H_2O$ to 20 µl, were incubated for 3 h at 37°C. Afterwards, the linearized plasmids were purified through Wizard® SV Gel and PCR Clean-Up System (Promega) as described

by the manual and quantified by NanoDrop 1000 (Thermo Scientific). The enzymatic digestions were checked on 1% agarose gel, comparing the running of linearized and circular plasmids (Figure 12). The circular plasmid, in fact, runs faster than the linearized due to a different conformation.

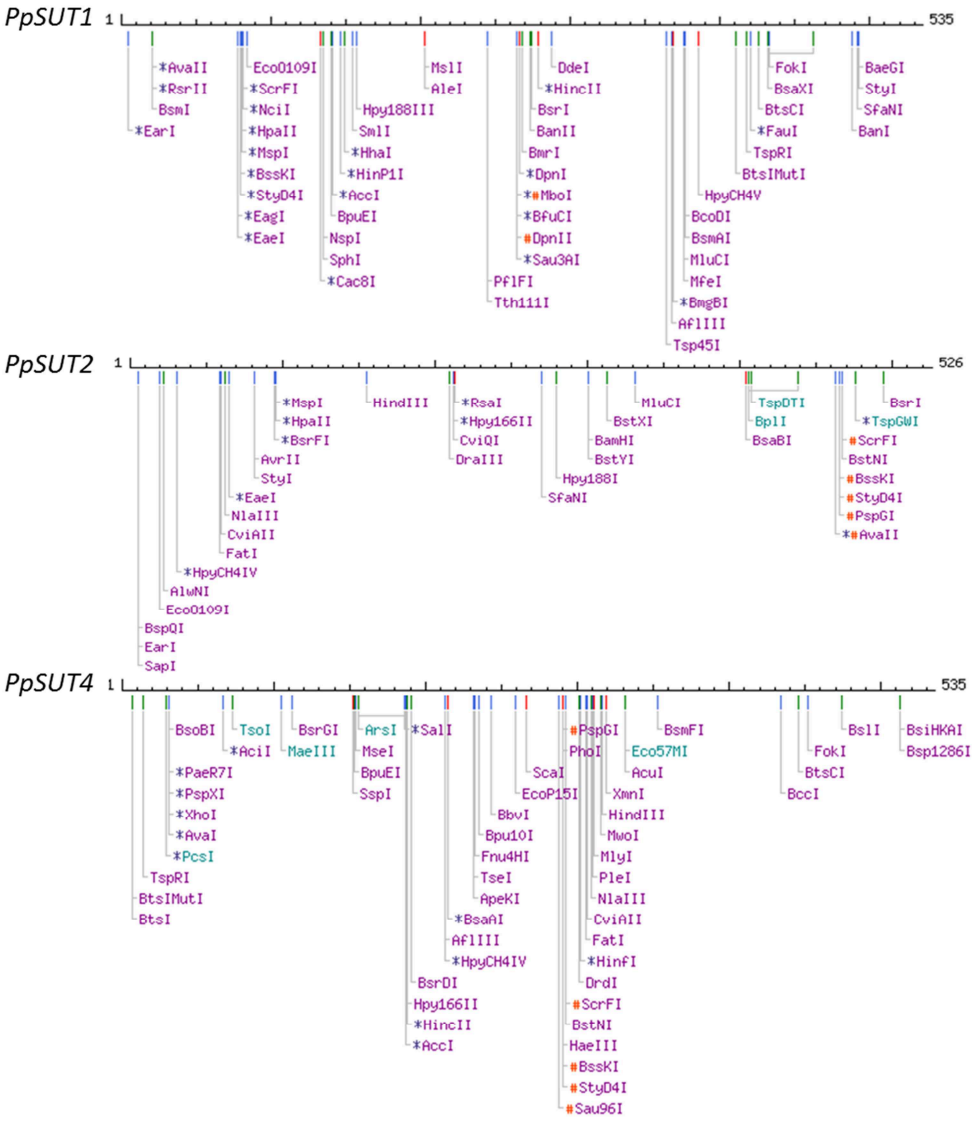


**Figure 13. Restriction enzyme map of *PpSUT1*, *PpSUT2* and *PpSUT4* obtained by NEB cutter web tool.**

The linearized vectors were used as a template for the synthesis of RNA probes in the presence of UTP conjugated with digoxigenin. The synthesis was carried out by DIG RNA Labeling Kit (Sp6/T7) (Roche). Two different RNA polymerase were used (T7 and Sp6 RNA polymerase) to produce both probe in sense and antisense directions. The reactions were prepared as follows: 1 µg of linearized plasmid, 2 µl 10X NTP labeling mixture, 2 µl 10X transcription buffer, 1 µl Protector RNase inhibitor, 2 µl RNA polymerase and $H_2O$ to 20 µl. The RNA polymerase was combined with the

linearized plasmid cut to the opposite side of the promoter recognized. The mixes were incubated 2 h at 37°C and subsequently treated with DNase I adding 2 µl to the solution and incubating 15 min at 37°C. The DNase action was stopped with the addition of 2 µl of 0.2 M EDTA (pH 8.0). Finally, the fresh DIG-RNA probes were cleaned by Rneasy Minelute Cleanup Kit (Qiagen) according to the manufacturer's instructions, quantified by NanoDrop 1000 (Thermo Scientific) and stored at -80°C.

### 2.2.14. Detection of *PpSUT* transcripts

A protocol of *in situ* hybridization specific for peach fruit was developed according to Garcês and Sinha (2009) with some modifications. Several parameters, such as incubation times, solutions concentrations (HCl, proteinase K, RNA probe), temperatures (hybridization and post-hybridization) were tested to find the best condition for a specific reaction. For each gene analyzed, two slides were prepared with consecutive tissue sections in order to treat one with antisense riboprobe and the other with sense probe, as a negative control for the specificity of hybridization reaction.

**TISSUE PREPARATION**

Paraffin-embedded samples were cut into 8 µm thick slices by means of the microtome Leitz 1512, placed onto positive charged slides, and incubated overnight at 37°C. Sections were de-waxed in limonene for 20 min (two changes for 10 min each) and rehydrate through serial steps in ethanol solutions at decreasing concentration (100%, 95%, 70%, 50% and 30%) followed by two washings in $H_2O$ DMDC. All steps were performed for 2 min.

**PREHYBRIDIZATION**

Tissues were immersed in 2X SSC (0.3 M NaCl, 0.03 M Trisodium citrate dehydrate, pH 7) for 20 min, then they were permeabilized by a treatment in 0.2 M HCl for 20 min followed by two washing in $H_2O$ DMDC for 2 min each. Then, specimens were incubated in TE buffer (100 mM Tris-HCl pH 8, 50 mM EDTA) containing 0.1 µg/ml proteinase K for 30 min at 37°C to perform a second step of permeabilization, after which they were transferred for 2 min in 1X PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.4) with 0.2% glycine added to stop the action of proteinase K. Hence, slides were washed twice in 1X PBS for 2 min, then transferred in 1X PBS with 4% paraformaldehyde for 10 min, followed by other two washing in 1X PBS for 2 min. In

order to reduce the background staining, a pre-treatment with 0.25% acetic anhydride and 0.1 M triethanolamine for 10 min in slow agitation was performed. After washing in 1X PBS for 5 min, sections were dehydrated through a serial steps in ethanol solutions at growing concentration for 30 sec each (30%, 50%, 70%, 95%, twice 100%), then left uncovered to allow the evaporation of ethanol. Afterwards, a step of pre-hybridization was carried out treating samples with hybridization buffer without probe for at least 1 h at 40°C in a humid chamber.

**HYBRIDIZATION**

The hybridization solution (40% deionized formamide, 4X SSC, 1X Denhardt's solution [0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 0.02% Bovine Serum Albumin], 0.1 mg/ml yeast tRNA, 5 mM DTT, 10% dextran sulfate) was incubated for 10 min at 75°C; then 0.1mg/ml of denatured salmon sperm and 5ng/µl of RNA labeled probe were added and the solution was incubated again at 75°C for 5 min. Subsequently, some drops of hybridization buffer were put onto tissue sections, slides were heated for 5 min at 65°C and incubated overnight in a humid chamber at the hybridization temperature, that was calculated for each probe with the following formula: $T_{hyb}$ = 24.21 + 0.41 (%GC) – 500/n (www.GeneDetect.com), where $T_{hyb}$ is the temperature of hybridization, %GC is the percentage of G or C in the probe, and n is the length of probe.

**POST-HYBIDIZATION**

The hybridization buffer was removed and slides were washed twice in 0.2X SSC for 1h at 40°C; then they were transferred in 1X PBS solution for 10 min and two times in BM block solution (1% Blocking reagent [Roche] in maleic acid buffer [100 mM maleic acid, 150 mM NaCl, pH 7.5]) for 45 min and 30 min, respectively. Hence, specimens were incubated in block solution 2 (1% Bovine Serum Albumin, 3% NaCl, 0.3% Triton-X 100, 10% Tris-HCl pH 7.5) for 45 min, after which they were drained. The hybrid mRNA/DIG-RNA probe was detected through an antibody anti-DIG alkaline phosphatase conjugate (Roche). The antibody was diluted 1:500 in block solution 2 and some drops of this solution were placed onto tissue sections; samples were incubated for 2 h, hence washed two times in block solution 2 for 20 min each and maintained in block solution 2 overnight at 4°C.

**DETECTION**

Specimens were transferred in a new block solution 2 and incubated for 20 min at room temperature, at the end of which they were washed in buffer C (0.1 M NaCl, 0.1 M Tris-HCl pH 9.6, 0.05 M $MgCl_2$) two times for 15 min and 10 min. Subsequently, they were drained from buffer C and some drops of color solution (1X NBT/BCIP (nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt) [Roche], 1 mM Levamisole ([-]-tetramisole hydrochloride, a suppressor of endogenous phosphatase activity), in buffer C) were put onto the tissue sections. Samples were incubated until color development at room temperature in a dark chamber. In effect NBT/BCIP yields an intense, insoluble black-purple precipitate when reacts with alkaline phosphatase. Before detecting the signal, color solution was removed and specimens were washed in buffer 3 (10 mM Tri-HCl pH 8, 10 mM EDTA) for 2 min and washed twice in $H_2O$ DMDC for 5 min. After drying, some drops of mounting (CC/Mount$^{TM}$ from Sigma) and a cover-slide were placed on the sections. Finally, samples were observed by optical microscope (B-350 Optika microscopes Italy).

## 2.3. Results

### 2.3.1. Fruit growth curve

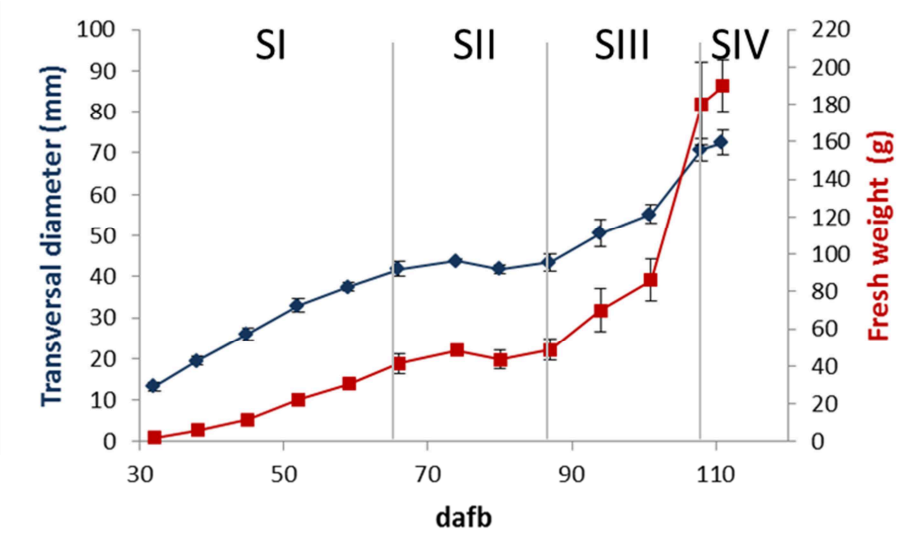


**Figure 14. Fruit growth of cultivar 'Redhaven' expressed as transversal diameter (in blue) and fresh weight (in red) during development (expressed as days after full bloom [dafb]).**

The plotting of fruit size or fruit weight against time has allowed to analyze the pattern of peach fruit growth, showing, in both cases, a double-sigmoid trend (Figure 14), characteristic of the Drupaceae species. In detail, four stages can be distinguished: two steps of rapid growth rate, from flowering to 66 dafb (SI), and from 87 to 108 dafb (SIII), interrupted by a lag phase from 66 dafb to 87 dafb (SII), and a final stage of growth slowdown from 108 dafb to harvest (SIV), that took place at 111 dafb.

### 2.3.2. Carbohydrates content

As expected, sugar quantification during development of Redhaven peach fruit showed a different accumulation pattern in relation to the type of carbohydrate considered. In detail, three main sugars were present, i.e. glucose, fructose and sucrose (Figure 15). In the early period of fruit growth, the two hexoses were the most abundant soluble sugars in mesocarp tissue. They showed similar levels throughout fruit growth, reaching a maximum around 50 dafb, and then a constant decreasing pattern until harvest. On the contrary, sucrose was fairly not present in young peach fruits, but begun to be steadily accumulated starting from about 40 dafb until harvest, becoming the predominant soluble sugar in mature fruit.

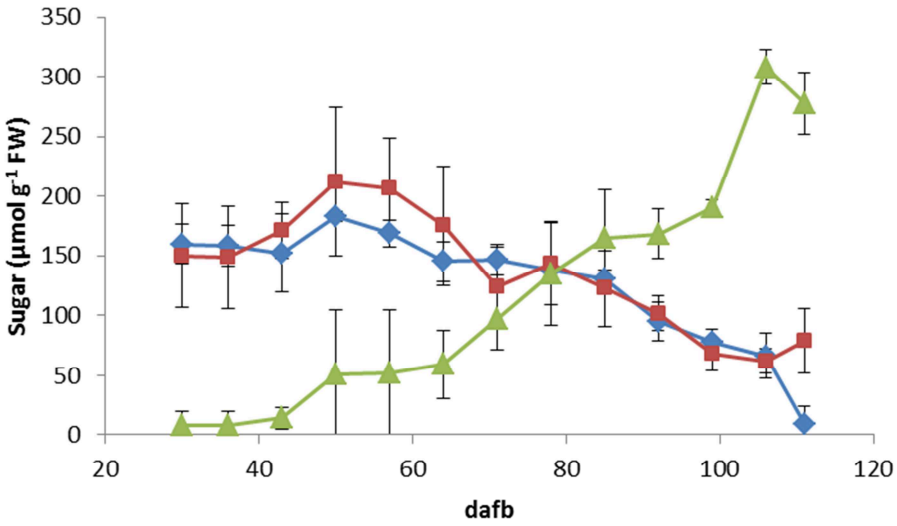


**Figure 15. Soluble sugars concentrations in mesocarp tissues of developing Redhaven peach fruit. Green line: sucrose; blue line: fructose; red line: glucose. Data represent mean values of three replicates, while bars denote ± standard deviation.**

### 2.3.3. Structure of peach fruit tissue

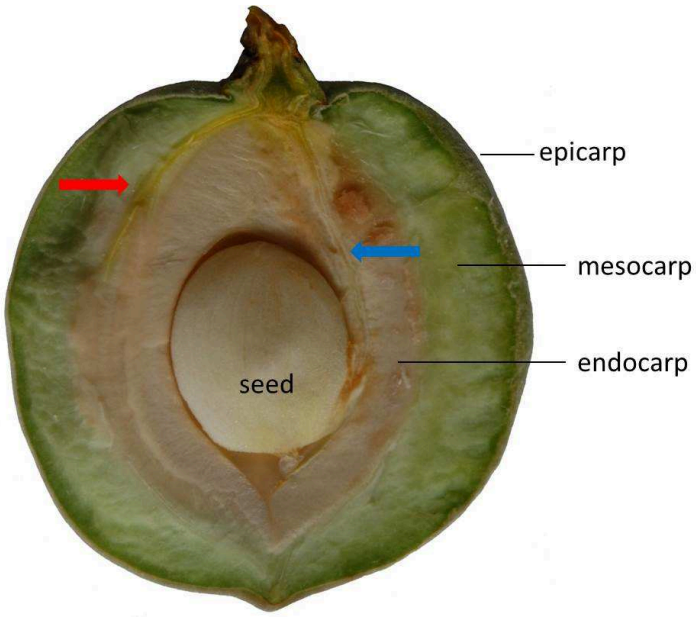


**Figure 16. Redhaven peach fruit (80 dafb). The arrows indicate the main vascular bundle, departing from the pedicel toward the seed (blue), and along the periphery of endocarp (red).**

Peach is a typical stone fruit characterized by an external skin (the epicarp), a fleshy pulp (the mesocarp), and a hardened stone (the endocarp) in which usually one seed is harbored (Figure 16).

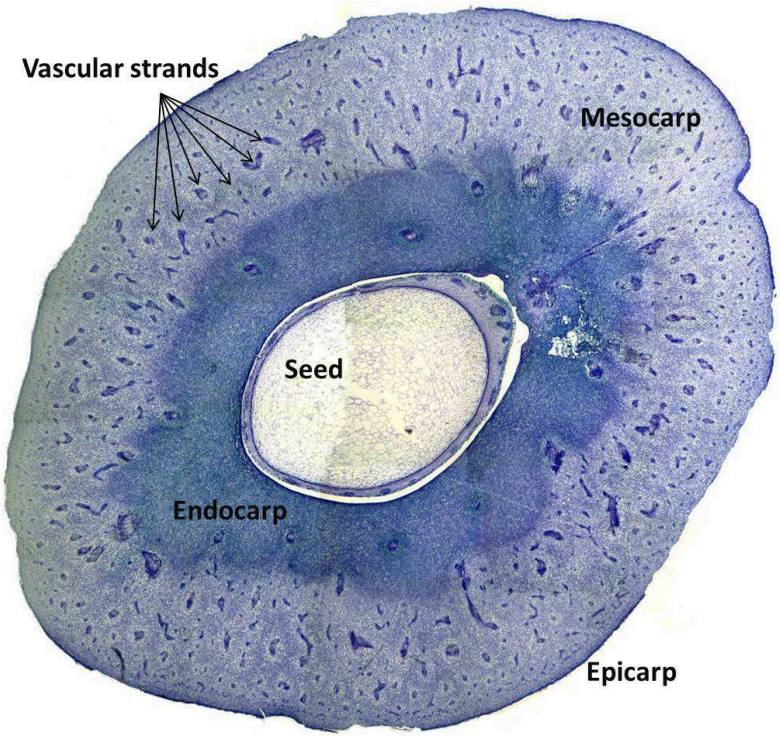


**Figure 17. Transverse section of a peach fruit during early period of development (about 5 mm diameter), stained with methylene blue.**

Inclusion and staining of young peach fruit, allowed to highlight the intricate network of vascular strands feeding the mesocarp tissue (Figure 17), whereas only few were detected in the endocarp, not still lignified. Moreover, the examination of vascular bundles at higher magnification revealed a slightly different structure according to the tissue localization. The ones present in the endocarp showed the typical vascular strand organization, consisting of lignified xylem cells and compact phloem cells (data not shown).

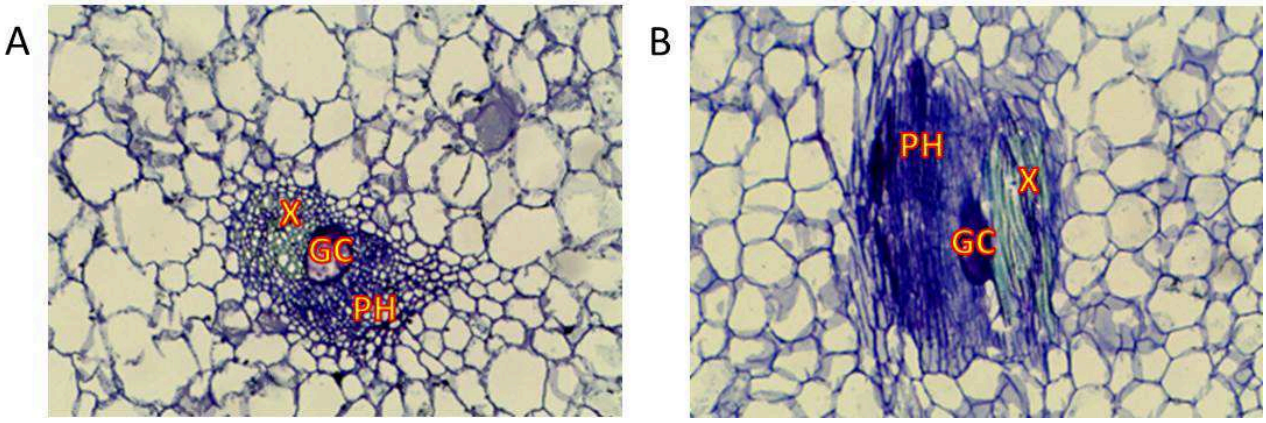


**Figure 18. Vascular bundle structure in the mesocarp tissue of peach fruit. X, xylem; PH, phloem, GC, glandular cavity. A: vascular bundle cut crosswise; B: vascular bundle cut longitudinally.**

Diversely, in the mesocarp, the majority of veins are composed of different structures: xylem cells, phloem cells and an additional portion constituted by a cavity located between the two types of conductive cells (Figure 18). This structure was previously described by Zhang *et al*. (2009) as a glandular cavity. The xylem cells were stained in light blue, while the phloem cells in dark blue. The big structure was observed in the central region.

### 2.3.4. Analysis of mechanism of phloem unloading by carboxyfluorescein

Assimilates transported toward sink organs by phloem system have to be unloaded into sink cells before utilization or storage. The phloem unloading can occur through two different pathways, symplasmic or apoplasmic, depending on the presence or absence of functional plasmodesmata, respectively. It has also demonstrated that the unloading route varies according to plant species, tissue and stage of development. Therefore, experiments were carried out on peach mesocarp tissues at three different growth stages by means of *in vivo* functional investigation of carboxyfluorescein distribution. This fluorescent tracer of phloem transport has been employed in order to examine the phloem sap movement within mesocarp.

To introduce the phloem tracer into the pericarp, peach branches carrying several leaves and one fruit at different growth stages were fed with an aqueous solution containing CFDA. Samples were successfully able to absorb and transport the fluorescent marker. The green fluorescence, observed by fluorescence microscope, was firstly detected in the pedicel zone, as shown in Figure 19. The pedicel is a region highly rich in vascular strands, for this reason the green signal detected was very bright and distributed in the entire section analysed.

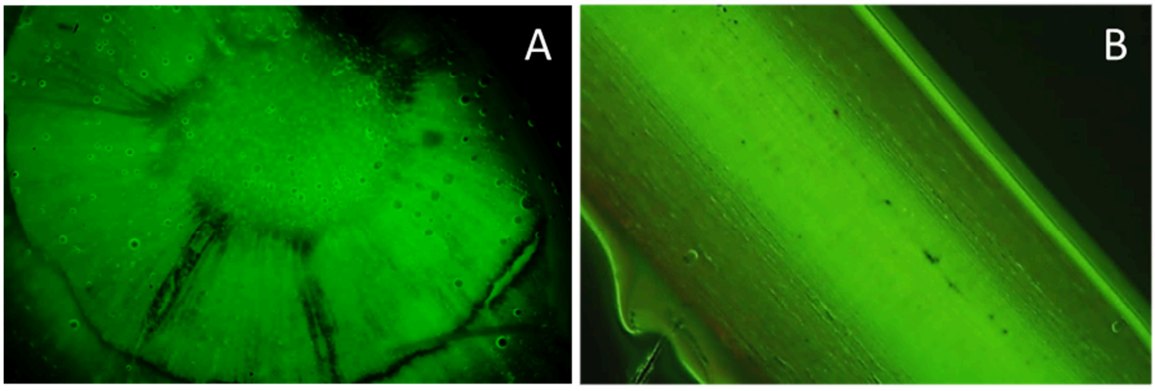


**Figure 19. Transversal (A) and longitudinal (B) hand sections of the pedicel region of peach fruit fed with aqueous solution containing CFDA. Pictures were taken by fluorescence microscope.**

However, when the sections of peach fruit were collected from regions more distant from the pedicel, the signal of CF started to be more localized. The vascular strands, in fact, depart from the pedicel and distributes more or less evenly throughout the sink organ, distancing from each other. This behaviour can be observed in Figure 20 which shows a section of an entire fruit treated during the early period of development, at about 45 dafb, when its diameter reaches approximately 5 mm.

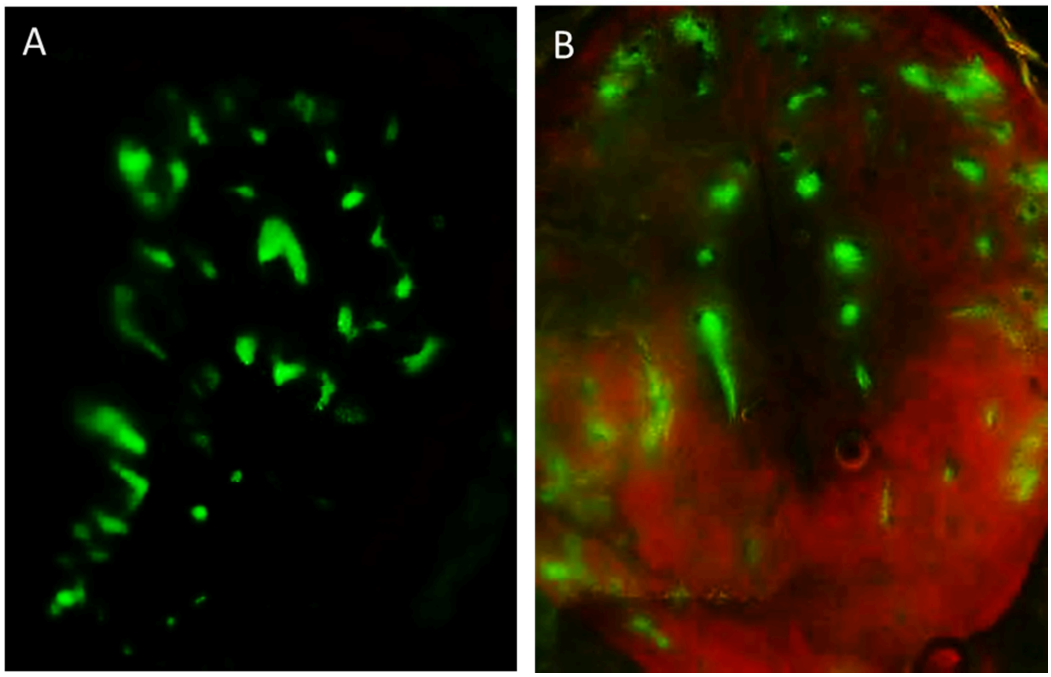


**Figure 20. CF distribution in whole peach fruits at early period of development (about 45 dafb). Pictures were taken by fluorescent microscope. A: fluorescence of CF restricted to phloem strands in a transversal section of peach fruit. B: fluorescence of CF circumscribed to vascular bundles in a longitudinal section of peach fruit. The red color represents the autofluorescence of chlorophyll.**

During this phase, the fluorescent marker remained confined almost exclusively to the phloem strands without apparent diffusion to the surrounding tissue. The vascular strands oriented in a different way are displayed in Figure 20A as irregular fluorescent spots spread in the entire transversal section of mesocarp. The localization of the signal mainly in the phloem tissue indicates the absence or the blockage of plasmodesmata conductivity, the only way that allows the diffusion of CF from SE/CC complex to adjacent parenchyma cells. Therefore, the movement of photoassimilates has necessarily to involve a step across the biological membranes. The longitudinal section of young peach fruit (Figure 20B) allowed to visualize that CF movement was unmodified in all its parts. In fact, in different portions of fruit, (pedicel side, central side and top side), fluorescence remained localized and confined into phloem cells, suggesting that the vascular

strands and the adjacent tissue are not interconnected. Moreover, also in the tissue that originates the endocarp the lack of fluorescence diffusion demonstrated the symplasmic isolation of sieve element.

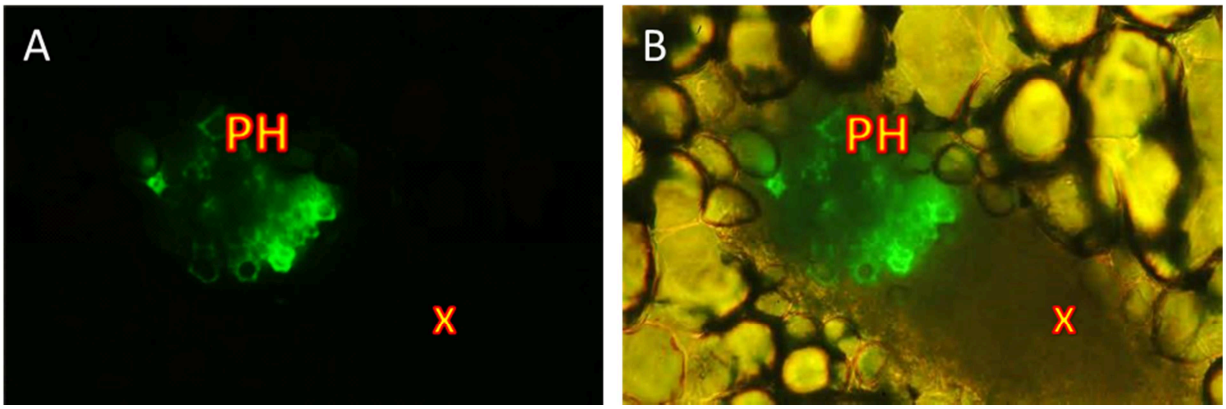


**Figure 21. Magnification of a vascular strand cut crosswise in peach mesocarp fed with a solution containing CFDA during the second stage of fruit development (about 75 dafb). A: fluorescence of CF circumscribed to phloem cells; B: fluorescence of CF together with transmission light. PH: phloem; X: xylem**

The transport of CF remained unaffected in fruits during the second phase of development, correspondent to a slowdown in fruit growth. As shown in Figure 21A, the green signal was detected specifically inside phloem cells. This result was confirmed by the observation of a longitudinal section of vascular strand (Figure 22), allowing to hypothesize an apoplasmic unloading also during the second stage of growth.

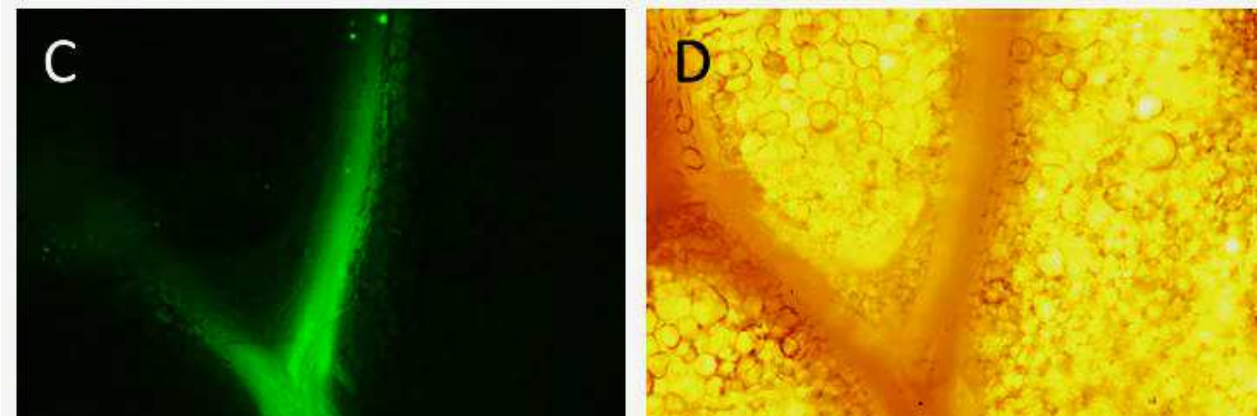


**Figure 22. Magnification of a longitudinal section of phloem strand in peach mesocarp during the second phase of fruit development (about 75 dafb). C: fluorescence of CF restricted to phloem cells; D: transmission light imagine.**

Instead, the symplasmic connection between SE/CC complex and parenchyma cells seemed to change in fruits near to ripening. In fact, at this stage, the CF was able to move from phloem cells to parenchyma cells, as shown by the release of fluorescence from the vascular bundle toward surrounding tissue (Figure 23). The green signal was more bright in the phloem tissues, but also noticeable inside bordering cells.

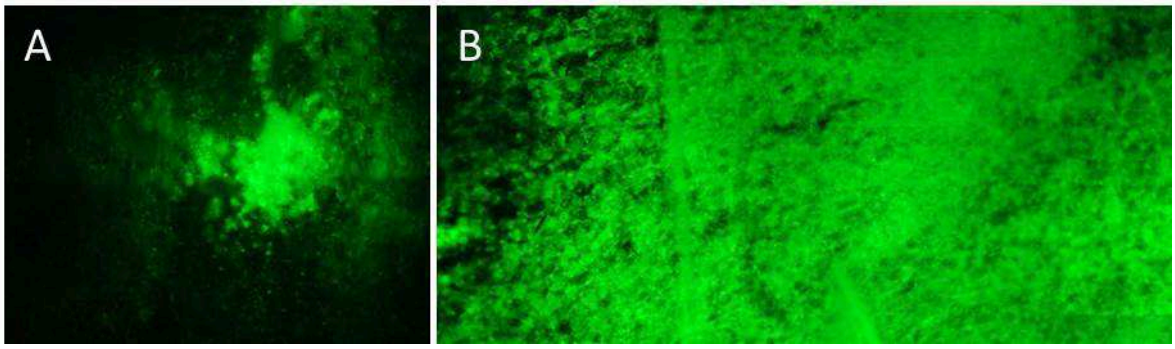


**Figure 23. CF distribution in mesocarp tissue, during the second rapid phase of fruit growth (about 105 dafb). A: diffusion of CF into tissue surrounding phloem strand; B: picture at higher magnification.**

### 2.3.5. Identification of genes encoding sucrose transporters in peach

Genes encoding sucrose transporters in *Prunus persica* were examined to understand their role in active sucrose transport within sink tissues.

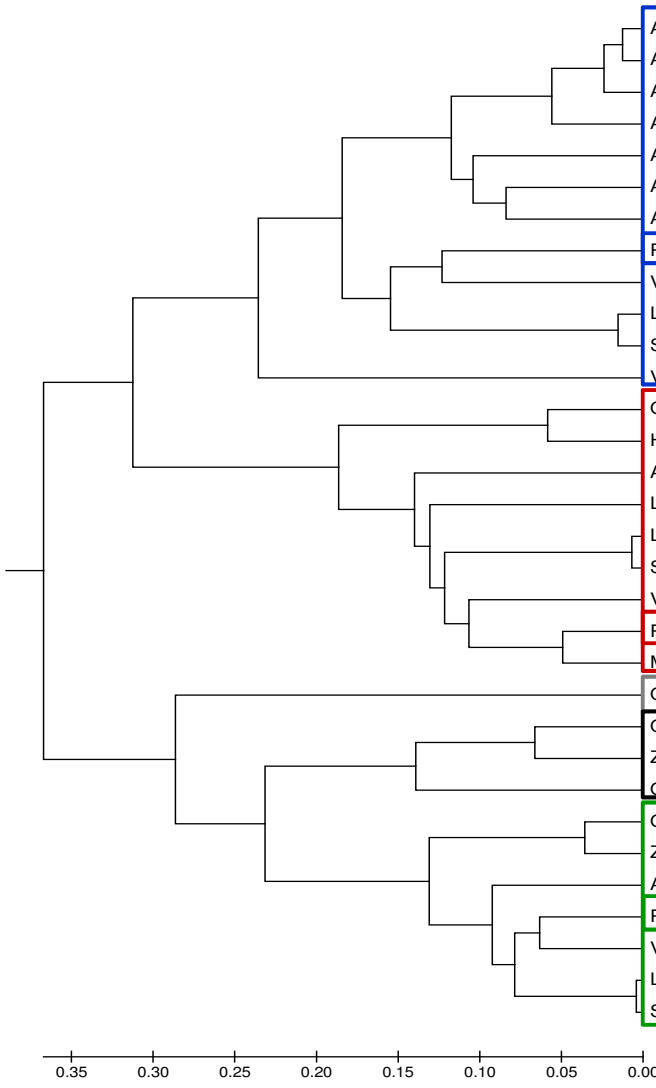


**Figure 24. UPGMA consensus tree of SUT proteins identified in peach and in other plant species. *Prunus persica*: PpSUT1 (ppa004033), PpSUT2 (ppa003041), PpSUT4 (ppa004620); *Arabidopsis thaliana*: AtSUC1 (At1g71880), AtSUC2 (At1g22710), AtSUC3/AtSUT2 (At2g02860), AtSUC4 (At1g09960), AtSUC5 (At1g71890), AtSUC6 (At5g43610), AtSUC7 (At1g66570), AtSUC8 (At2g14670), AtSUC9 (At5g06170); *Oriza sativa*: OsSUT1 (Os03g07480), OsSUT2 (Os12g44380), OsSUT3 (Os10g26740), OsSUT4 (Os02g58080), OsSUT5 (Os02g36700); Malus domestica: MdSUT1 (AAR17700.1); *Lycopersicon esculentum*: LeSUT1 (CAA57726), LeSUT2 (AAG12987), LeSUT4 (AAG09270); *Solanum tuberosum*: StSUT1 (CAA48915), StSUT2 (AY291289), StSUT4 (AAG25923); *Vitis vinifera*: VvSUT2 (AAL32020), VvSUC11 (AF021808), VvSUC12 (AF021809), VvSUC27 (AF0218010); *Lotus japonicus*: LjSUT4 (CAD61275); Zea mais: ZmSUT1 (NP_001104840), ZmSUT2 (NP_001146651); *Hordeum vulgare*: HvSUT2 (CAB75881); Rectangles were used to point out the 5 clades according to the classification of Kühn and Grof (2010). Blue: clade 1; green: clade 2; black: clade 3; red: clade 4; grey: clade 5.**

The availability of peach genome (http://www.rosaceae.org/species/prunus_persica/genome_v1.0) has allowed the identification of genes encoding sucrose transporter through BLAST search with SUT proteins from *Arabidopsis thaliana*.

At least three predicted genes were identified, named ppa004033, ppa003041, and ppa004620. Both ppa003041 and ppa004620 were located in pseudomolecule 1, while ppa004033 was positioned in pseudomolecule 8. Moreover, comparison of the deduced amino acid sequence of the three genes with peptide sequences of sucrose transporters from other plant species showed a difference in length only for ppa004033, indicating a putative error in the prediction. Furthermore, the comparison between transcript prediction and RNA-seq profile, available in the public genome browse (http://services.appliedgenomics.org/fgb2/iga/prunus_public/gbrowse/prunus_public/) showed a discrepancy at the beginning of the sequence and the attempt to amplify the whole ppa004033 CDS from cDNA with different primers and reaction conditions was unsuccessful (data not shown). For these reasons, a manual correction of the sequence was performed and another putative start codon was identified in the first intron predicted, 36bp upstream of the second exon. Moreover, the new initiation codon ATG identified presented a purine at positions −3 and +4, in accordance with the Kozak consensus sequence (Kozak, 1987).

Analysis of similarity with different SUT proteins from several plant species and the three putative sucrose transporters identified in peach is shown in Figure 24. According to Kühn and Grof (2010), the SUT proteins are classified in 5 clades. The three proteins from peach were grouped into the three clades belonging to the dicots, SUT1, SUT2 and SUT4 clades. The genes from *Prunus persica* were renamed *PpSUT1* (ppa004033), *PpSUT2* (ppa003041) and *PpSUT4* (ppa004620) in agreement with this classification.

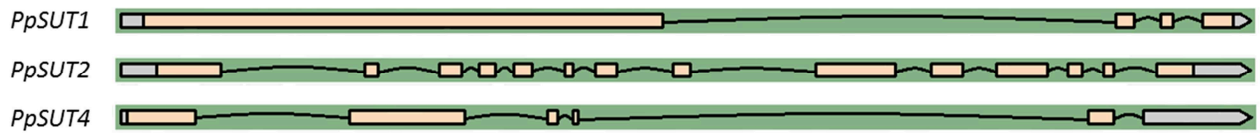


**Figure 25. *PpSUT* gene structures from *Prunus persica*. UTRs are represented as pale blue regions.**

Gene structures (Figure 25) were very similar from those of other plant species, in relation to clade membership (Shiratake, 2007). *PpSUT1* consisted of a large first exon followed by other three smaller, unlike other genes that belong to this group which have usually only two short final exons. *PpSUT4* comprised two large and three small exons. *PpSUT2*, instead, showed a very different structure with several small exons, as other members of SUT2 clade. The predicted proteins

resulted of 511, 609 and 499 amino acids residues for PpSUT1, PpSUT2 and PpSUT4, respectively. Their molecular mass was estimated of about 54 KDa for PpSUT1 and PpSUT4, and approximately 65 KDa for PpSUT2,. The putative amino acid sequences were analyzed by TMHMM Server v. 2.0 (Figure 26), a web tool of the Center for Biological Sequence Analysis that calculate the probability of a sequence to form a transmembrane domain (from 0 to 1). All three PpSUT proteins displayed 12 highly probable transmembrane regions, typical structure of the members of the major facilitator superfamily. Moreover, both N-terminal and C-terminal were localized in the cytosolic side. The most relevant difference between the three proteins relied on PpSUT2 characteristics, such as a more extended N-terminal and a large central hydrophilic loop, typical of the SUT2 clade members. Additionally, a dileucin-like motif (LXXLL), a specific sequence for vacuolar targeting (Aoki *et al*., 2003), was only found in the N-terminal cytoplasmic domain of PpSUT4 sequence (position 23-27, LRQLL).

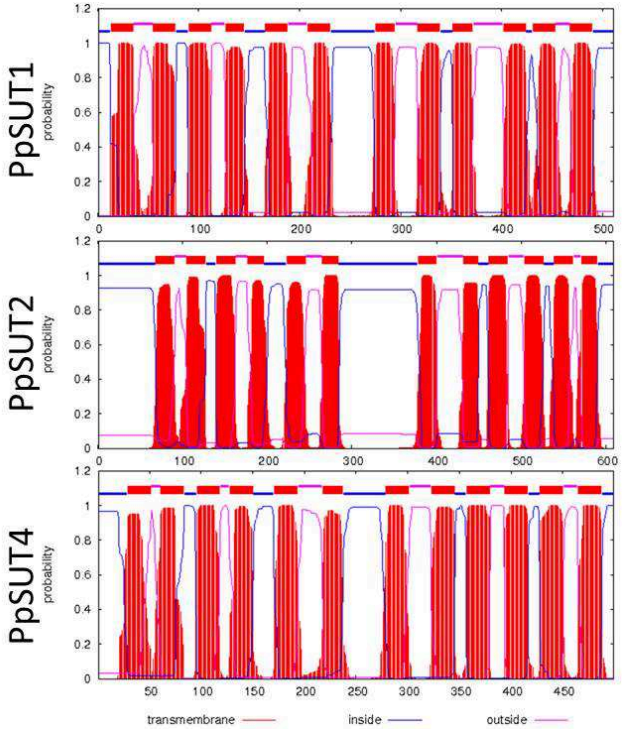


**Figure 26. Prediction of transmembrane domain by TMHMM Server v. 2.0 software for PpSUT1, PpSUT2 and PpSUT4. The web tool shows the probabilities (indicated on the y-axis, from 0 to 1) of a sequence to form inside/outside/transmembrane domains; regions predicted to be located inside or outside the membrane are shown in blue and pink, respectively, while domains predicted to be transmembrane are shaded in red.**

### 2.3.6. Analysis of PpSUT genes expression

The expression analysis of the *PpSUT* genes were performed by means of two different techniques: Real-Time PCR and *in situ* hybridization. In particular, Real-Time PCR was used either to analyse the transcripts level during the development of peach fruit, and, in combination with

laser microdissection pressure catapulting (LMPC), to compare the level of gene expression in two different types of cell, phloem and parenchyma cells. *In situ* hybridization was adopted to localize the transcripts in mesocarp tissue sections.

**Real-Time PCR**

The analysis of gene expression was carried out on samples collected at different development stages, starting from about 30 dafb to harvest, occurred at 111 dafb. The three genes exhibited different expression levels. In particular *PpSUT4* was found to be the most abundant transcript in fleshy tissue. Its expression was fairly modulated throughout development (Figure 27). In young fruits, transcripts levels were low, increased quite constantly until a maximum level occurred at 56 dafb, and after a reduction at 64 dafb, peacked at 70 dafb. Finally, in mature fruits charaterized by a high accumulation of sucrose, the relative abundance of *PpSUT4* exhibited a decrease up to the completion of fruit development, at 111 dafb. In contrast, both *PpSUT1* and *PpSUT2* displayed lower transcript levels, as compared with *PpSUT4*. *PpSUT1* was characterized by lack of expression (Figure 28), while the amount of *PpSUT2* transcripts was higher and peaked at 42 and 70 dafb (Figure 29).

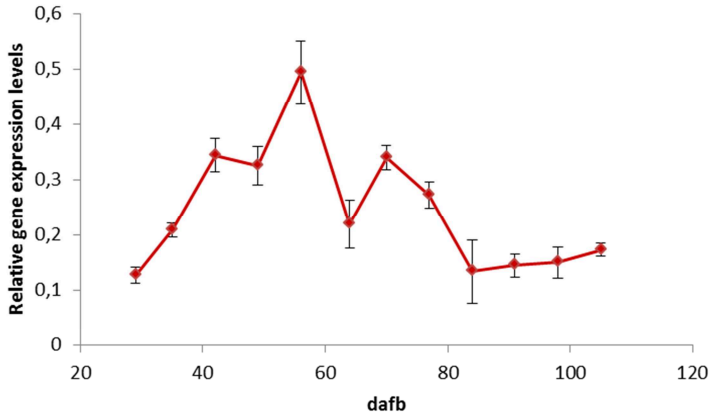


**Figure 27. Relative abundance of *PpSUT4* transcripts detected by Real-time PCR in peach mesocarp of cultivar Redhaven during development. The bars represent standard deviation calculated from the mean of three replicates.**

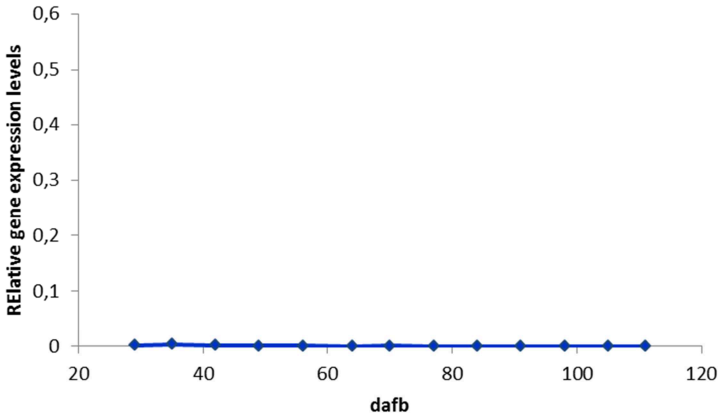


**Figure 28. Relative abundance of *PpSUT1* transcripts detected by Real-time PCR in peach mesocarp of cultivar Redhaven during development. The bars represent standard deviation calculated from the mean of three replicates.**

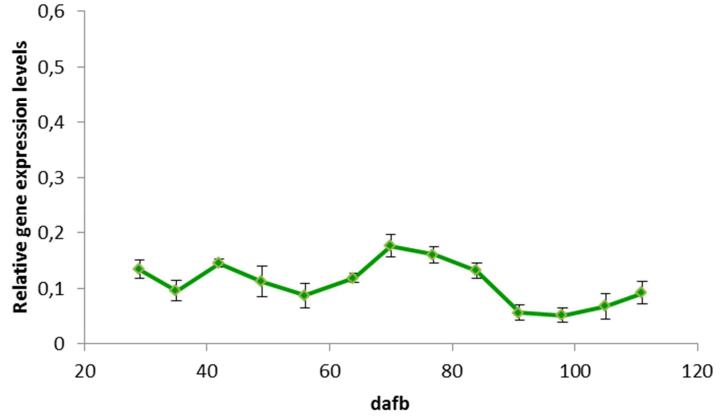


**Figure 29. Relative abundance of *PpSUT2* transcripts detected by Real-time PCR in peach mesocarp of cultivar Redhaven during development. The bars represent standard deviation calculated from the mean of three replicates.**

## Real-Time PCR – Laser microdissection pressure catapulting (LMPC)

The combination of two different approches, namely the laser microdissection pressure catapulting (LMPC) and the Real-Time PCR, allowed the quantification of *PpSUT* transcripts in two distinct cells types, phloem and parenchyma cells. Differences in the relative expression levels were observed in two stages of fruit development. In immature fruits both *PpSUT2* and *PpSUT4* were detected in phloem cells (blue color in Figure 30), but with variations in the levels. *PpSUT2* showed the highest expression, *PpSUT4* almost three times lower than *PpSUT2*, while *PpSUT1* was practically undetectable. Differently, in parenchyma cells (red color in Figure 30) transcripts of *PpSUT2* and *PpSUT4* were present in nearly equal amount, whereas *PpSUT1* was not detected.

*PpSUT2* was found with higher expression mainly in phleom calls, whereas *PpSUT4* in parenchyma tissue.

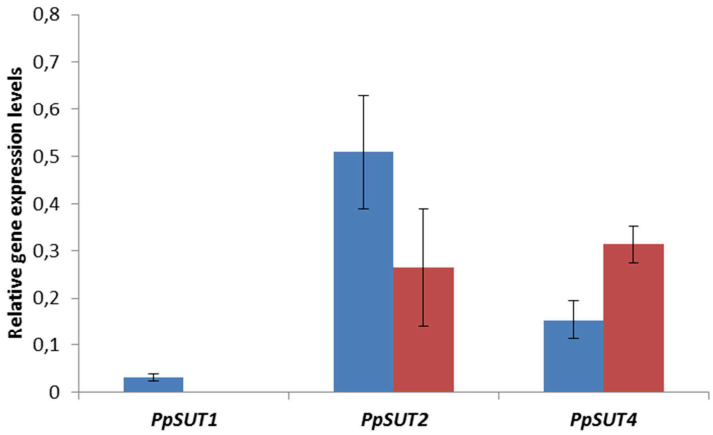


**Figure 30. Relative gene expression levels of *PpSUT1*, *PpSUT2* and *PpSUT4* measured in two different cell types, phloem cells (blue) and parenchyma cells (red) by combination of LMPC and Real Time PCR in young fruits (50 dafb). The bars represent standard deviation calculated from the mean of three replicates.**

When analyzed in fruit peach during the second stage of development, *PpSUT* genes were differently expressed. Firstly, the transcripts level of all *PpSUT* genes were much lower than those measured in immatue fruit. *PpSUT4* transcript was the unique found in parenchyma cells, even if it continued to be expressed in both cell types (Figure 31). On the contrary, *PpSUT1* was undetectable in both cell types, whereas *PpSUT2* was found only in phloem cells.

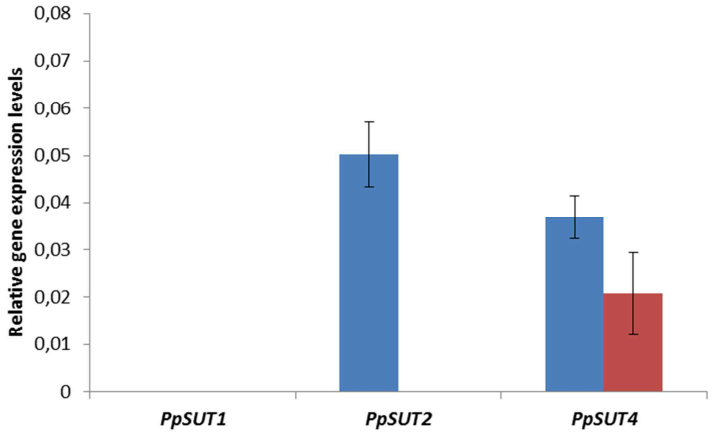


**Figure 31. Relative gene expression levels of *PpSUT1*, *PpSUT2* and *PpSUT4* measured in two different cell types, phloem cells (blue) and parenchyma cells (red) by combination of LMPC and Real Time PCR in fruits collected during the second phase of development (85 dafb). The bars represent standard deviation calculated from the mean of three replicates.**

### *In situ* hybridization

To validate the results of transcripts quantification in different cell types, *PpSUT* transcripts were localized in mesocarp tissue by means of *in situ* hybridization experiments. For each gene, two different RNA probes labeled with digoxigenin were designed in antisense and sense direction, the latter with the aim of ensure the specificity of the hybridization. The successful interaction between antisense probe and mRNA was observed trough a colorimetric assay. The expression of the three *PpSUT* genes was evaluated in serial sections of the same sample in two different phase of development.

The analysis conducted in immature peach mesocarp, with all probes, (Figure 32) displayed a distinct staining, in particular in the phloem tissue. The comparison between section treated with sense and antisense probe of all three genes allowed to identify a staining of parenchyma cells, looking more dark in section treated with antisense probe. In plant cells, most of the cell volume is occupy by the vacuole. As a consequence, the cytoplasm, and hence the transcripts, are set against the plasma membrane. For these reasons, it's possible to hypothesize that all the three probes interacted also with transcripts in the parenchyma cells

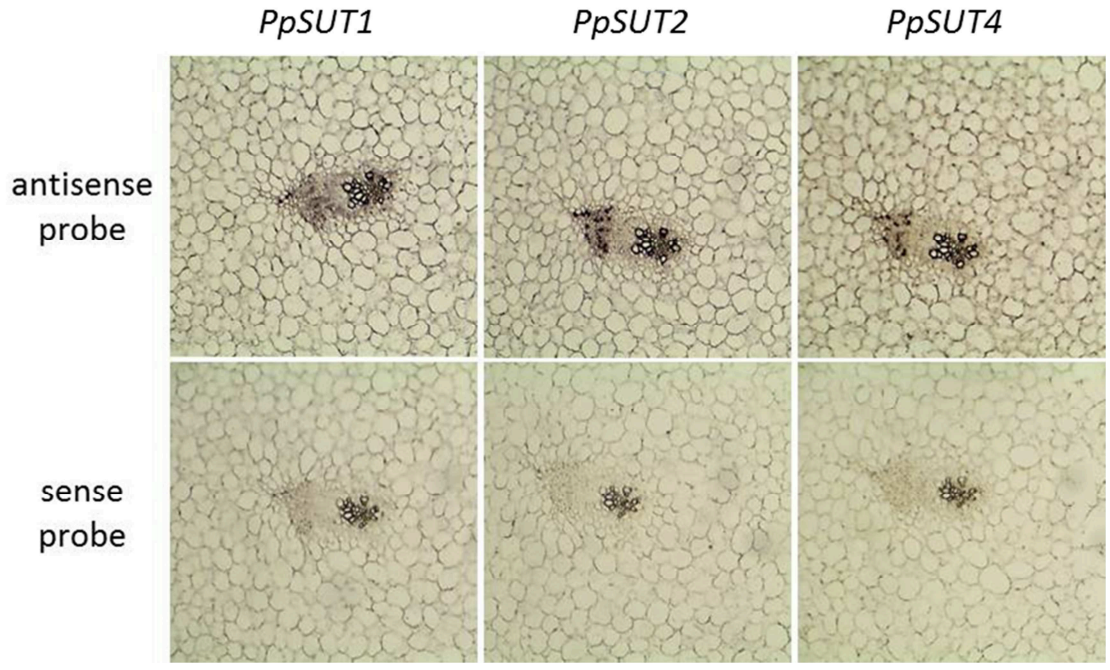


**Figure 32. Gene expression localization of *PpSUT1*, *PpSUT2* and *PpSUT4* in serial sections of mesocarp tissue of immature peach fruit (50 dafb) by means of *in situ* hybridization.**

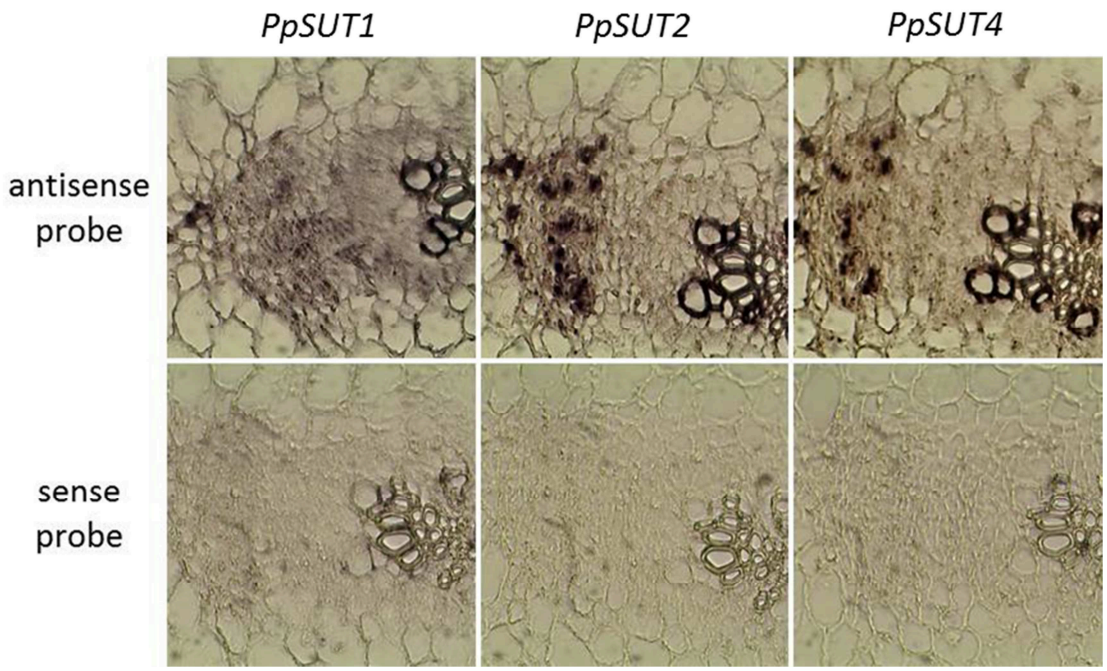


**Figure 33. High magnification of phloem strand in mesocarp tissue of immature peach fruit (50 dafb) treated with *PpSUT1*, *PpSUT2* and *PpSUT4* probes.**

However, the most prominent difference was detected in phloem tissue. This evidence is observed better at higher magnification (Figure 33). Mesocarp sections incubated with *PpSUT1* antisense probe led to slight and spread staining of phloem tissue. Differently, the localization of *PpSUT2* and *PpSUT4* transcripts was highly circumscribed in specific cells constituting phloem tissue, as shown by the dark and well defined spots, not detected in the control. The hybridization conducted on mesocarp tissues of samples collected during the second phase of fruit development revealed a change in *PpSUT* gene expression (Figure 34). In fact, labeling of parenchyma cells was apparently detected only in section treated with *PpSUT4* probe. Additionally, the spots previously localized in phloem cells in samples treated with *PpSUT2* and *PpSUT4*, disappeared, and only a slight staining was observed (Figure 35). The result indicated a general decrease in gene expression levels.

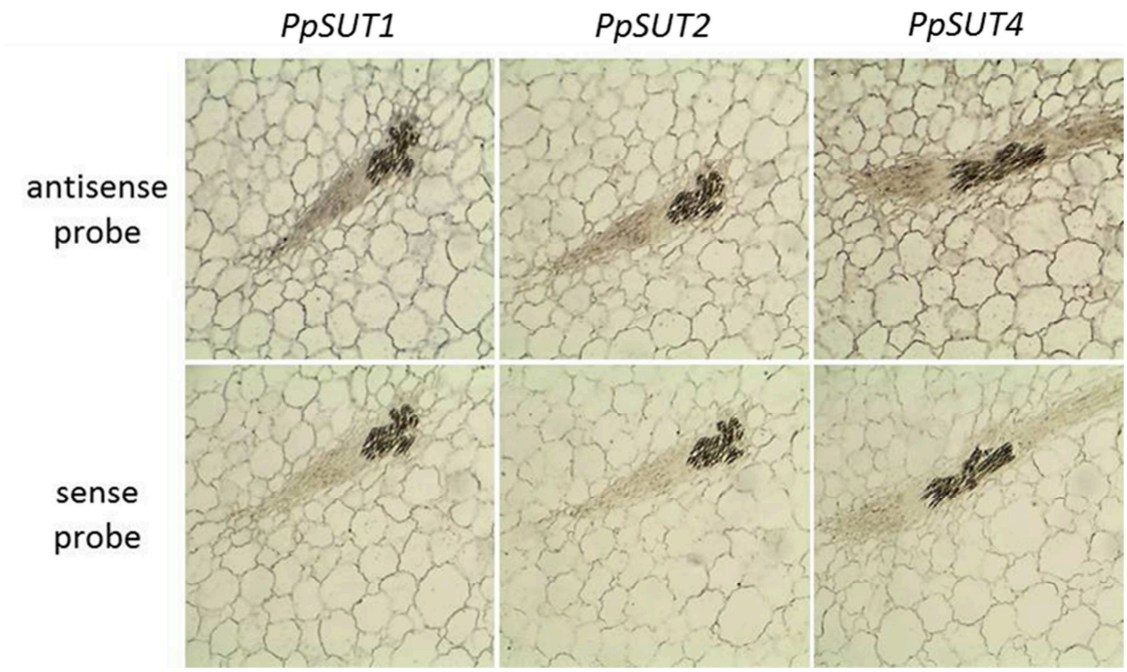


**Figure 34. Gene expression localization of *PpSUT1*, *PpSUT2* and *PpSUT4* in serial sections of mesocarp tissue collected during the second stage of fruit development (85 dafb) by means of *in situ* hybridization.**

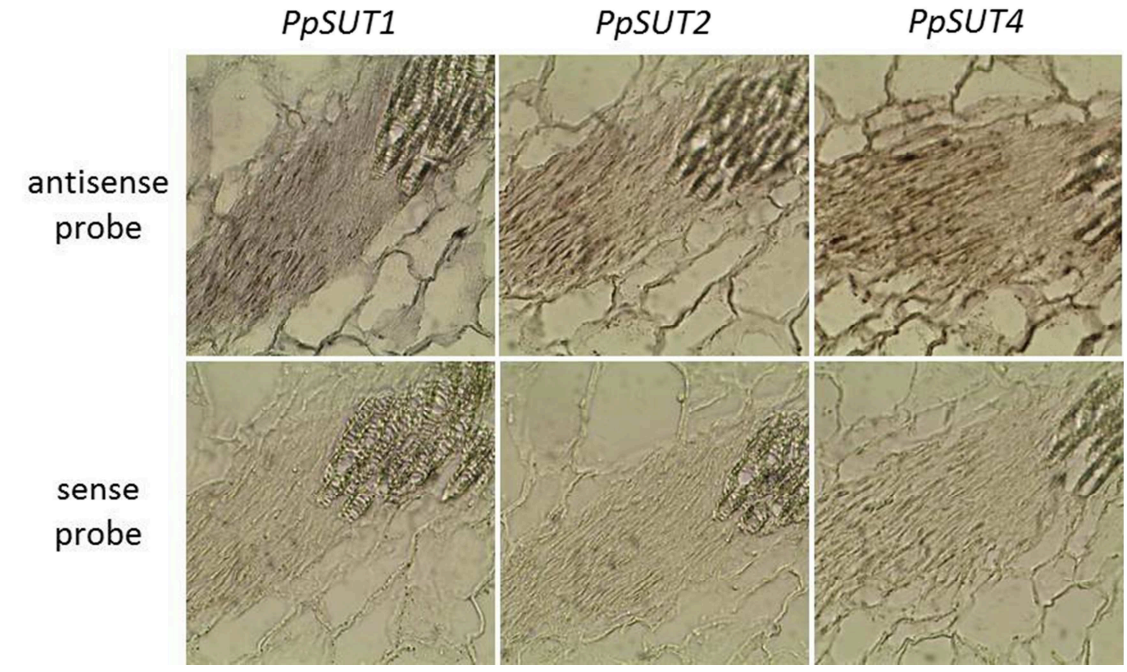


**Figure 35. High magnification of phloem strand in mesocarp tissue of fruit during the second development stage (85 dafb) treated with *PpSUT1, PpSUT2* and *PpSUT4* probes.**

## 2.4. Discussion

The mechanism of phloem unloading and post-phloem transport has received much consideration as part of the set point regulating assimilates partitioning. If the phloem transport is not source-limited, the efflux and accumulation of sugars in sink organs depends on several processes, such as phloem unloading, post-phloem transport, metabolic conversion, and compartmentation (Yamaki, 2010). Particularly, phloem unloading plays a key role in maintaining a high hydrostatic pressure gradient between source and sink, favorable to phloem sap movement, and in regulating the relationships among competing sinks in the plant (Herbers and Sonnewald, 1998). Two distinct routes for photoassimilates release have been described, the symplasmic and apoplasmic pathway. In the first case, carbohydrates move from one cell to another through a process driven by passive diffusion, due to the presence of plasmodesmatal connection between cells. On the contrary, when symplasmic continuity is absent, sugars are actively transported across biological membranes by sugar carriers.

In the recent years, assimilates partitioning has been analyzed in a great number of important storage sinks, including fruits. In these organs, carbohydrates fulfill different tasks. Firstly, they represent the main source of carbon and energy essential for growth and development. Additionally, their accumulation into vacuole stimulates influx of water into the storage organelle, and consequently determines the enlargement of parenchyma cell, leading, to an increase in fruit size (Yamaki, 1984). Carbohydrates are also key signaling molecules which can potentially regulate cell division, growth, and metabolism (Koch, 1996; Smeekens, 2000). Finally, the concentration and types of soluble sugars are of paramount importance in determining fruit quality (Colaric *et al*., 2005; Song and Forney, 2008; Seymour *et al*., 2013).

As far, researches on phloem unloading in fruits have highlighted a complex pattern according to genotype, stage of development, and tissue.

No information are still available for peach, the third most important fruit species after apple and pear in the world market (Byrne *et al*., 2012). As others stone fruits, peach exhibits a double sigmoid growth kinetic, characterized by four stages of growth. The first, and the third phases show a rapid increase in fruit size determined mainly by active cell division, and cell enlargement, respectively. During the other two phases, fruit growth rate is significantly reduced. In particular, the growth slowdown in the second phase is related to the endocarp lignification, embryo development, and accumulation of reserves into the seed (Bonghi *et al*., 2011; Falchi *et al*., 2013).

Each developmental stage is also characterized by a distinct, and dynamic pattern of sugar accumulation. In immature fruits, in which rapid cell division takes place, the main sugars were glucose and fructose, consistently with the hypothesis of a link between hexoses and mitotic activity (Weber *et al*., 1996). On the other hand, sucrose, probably involved in stimulating cell differentiation (Weber *et al*., 1996), accumulates during the final phases of fruit development. Interestingly, the switch in hexose:sucrose ratio occurs during the second phase of slow fruit growth.

The accumulation of sugars within fruit is a process strictly connected to phloem transport. In fact, even if young peach fruit photosynthesizes, the contribution in term of fixed carbon is limited to 5-9% (Pavel and DeJong, 1993). As a consequence, most part of sugars imported in fruit derives from phloem system. In young peach fruit section, phloem appears to form a network of veins. A great number of vascular strands are present in mesocarp, whereas only few are detectable in the tissue that originates the endocarp. As a common trait of Rosaceae species, in *Prunus persica*, besides sucrose, also sorbitol plays a role as a form of translocated carbon (Moing *et al*., 1997). Presently, it is still unclear if peach preferentially transports sorbitol or sucrose in the phloem (Layne and Bassi, 2008). Some authors have postulated an utilization of sorbitol in vegetative sink tissues close to the source, such as young sink leaves and cambium (Moing *et al*., 1992), and a major role of sucrose in fruit growth (Lo Bianco *et al*., 1999). The first attempt to understand sugar accumulation in peach was achieved through the analysis of protein activity of the key enzymes controlling the sucrose and sorbitol metabolism (Moriguchi *et al.,* 1990). Several studied, performed on different cultivars, revealed distinct regulation according to the genotype. However, analysis carried out on Redhaven peach fruits, revealed that enzymes related to sucrose degradation are mainly active in young peach fruit, and those controlling sucrose synthesis are rather inactive in mature fruit. For this reasons, it was postulated an accumulation of sucrose depending mainly on the direct import of disaccharide into the fruit (Vizzotto *et al*., 1996).

To investigate the mechanism of photoassimilates unloading in peach mesocarp, the movement of a fluorescent tracer of phloem transport (carboxyfluorescein, CF) was analyzed during fruit development. Results show that in peach fruit, as in other plant species, the pathway of phloem unloading changes according to development stage. In detail, it shifts from apoplasmic to symplasmic, the turning point being at or just before the dramatic increase in sucrose accumulation. The involvement of an apoplasmic pathway during early period of peach fruit development was previously hypothesized (Vizzotto *et al*., 1996). The symplasmic pathway has

been associated with lower resistance and greater transport capacity, as compared with apoplasmic process (Patrick, 1997; Patrick and Offler, 1996). Therefore, peach fruits might adopt predominantly the symplasmic route in the last phases of growth to transport high amount of sucrose. However, because no sucrose degradative enzymes have been detected in Redhaven mature fruit (Vizzotto *et al*., 1996; Nonis *et al*., 2007), the disaccharide has to be rapidly removed from the cytosol into the vacuole, in order to maintain a concentration gradient between phloem and parenchyma cells, favorable the movement of assimilate within sink cells. However, the uptake of sucrose into the storage organelle against its concentration gradient assumes the presence of an $H^+$/antiporter, even if this protein has not been yet identified.

The apoplasmic step, postulated to occur in greater extent during early and middle stages of fruit development, requires the involvement of sugar transporters to mediate the movement of carbohydrates across biological membranes. In this contest, become relevant the analysis of protein involved in the transfer of sugars from one compartment to another. Since previous works suggested a major role of sucrose than sorbitol in the supply of peach fruit, the analysis was specifically directed to the study of sucrose transporters. Three putative genes were identified through blast search on peach genome with SUT proteins from Arabidopsis, clustered into the three clades of SUT proteins from dicotyledonous, following the classification adopted by Kühn and Grof (2010), and consequently renamed according to clade membership. Analysis of hydrophobicity predicted 12 highly probable transmembrane regions, common structure of members of the major facilitator superfamily, and both C-terminal and N-terminal were localized in the cytosolic side. Moreover, PpSUT2 exhibited a longer N-terminal and larger central hydrophilic loop, as other members of SUT2 clade, while PpSUT4 presented a dileucin-like motif at N-terminal that has been identified as specific sequence for vacuolar targeting, not present in the other two proteins.

In this study, the first approach adopted to understand the role of sucrose transporters in the distribution of assimilate within peach fruit has been the analysis of gene expression throughout development. The three *PpSUT* genes show different expression patterns, suggesting a distinct and specific function in carbohydrates allocation into sink cells. *PpSUT4* displays the most abundant transcript level during development. Other members of SUT4 subfamily have been found in fleshy fruit, such as *MdSUT1*, *PbSUT1*, and *VvSUC11* suggesting an involvement of these proteins on sugar distribution (Zhang *et al*., 2013; Peng *et al*., 2011; Davies *et al*., 1999). Several members of this subfamily have been localized in the tonoplast. The identification of a dileucin-

like motif, a specific sequence for vacuolar targeting (Aoki *et al*., 2003), argued for a vacuolar localization of PpSUT4, in order to regulate the release of sucrose from the vacuole into the cytosol.

On the contrary, *PpSUT2* mRNA is present in very low amount during fruit development, consistently with the behavior of other members belonging to SUT2 subfamily in numerous sink tissues (Ayre, 2011). Genes belonging to this family have been detected in fruit, as the sink specific sucrose transporter *VvSUC12* from *Vitis vinifera*, together with *VvSUC11* (SUT4 members), showed to be upregulated in berries at the beginning of hexose accumulation (Davies *et al*., 1999; Ageorges *et al*., 2000; Manning *et al*., 2001).

The sucrose transporters belonging to SUT1 subfamily, have been generally identified in source organs, with some exception, i.e. in grape, *VvSUC27* was specifically expressed in early phase of berry development, while during hexose accumulation the transcript level decreased (Davies *et al*., 1999; Ageorges *et al*., 2000; Manning *et al*., 2001). *PpSUT1* was not detected in mesocarp tissue, therefore it is suggested to play a minor role in peach sucrose distribution.

According to CF and gene expression results, the following model can be proposed. During the early period of development, fruit growth depends mainly by cell division. Phloem and sink cells are symplasmically isolated. Therefore, sucrose is unloaded by an apoplasmic mechanism, and released into the apoplast. Because high invertase activities, soluble and insoluble, have been detected in immature fruit (Vizzotto *et al*., 1996; Nonis *et al*., 2007), sucrose may be uptaken from the apoplast either directly and as hexose, by the activity of sucrose and hexose transporters, respectively. However, the levels of *PpSUT2*, possibly located at the plasma membrane, remain low, and hence an existence of a further sucrose transporter playing this role is hypothesized. The products of invertase activity are directly used to sustain metabolism. In fact, in young fruit, the levels of hexose are high. As fruit grows, sucrose starts to be gradually accumulated, then in part have to be transferred into storage organelle. Therefore, it is suggested the presence of an $H^+$/antiporter, even if this protein has not been yet identified, that rapidly uptakes sucrose into the storage organelle against its concentration gradient. The levels of *PpSUT4*, supposed to be a vacuolar transporter, increase and remain higher also during the second phase of development, characterized by a shift of hexose:sucrose ratio. Since sucrose is rapidly stored into the vacuole, *PpSUT4* could have a role in the regulation of sucrose release to sustain cell metabolism. At the end of the second phase of fruit development, the unloading of sucrose changes between an apoplasmic to a symplasmic mechanism. Sucrose, reaching sink cells through the plasmodesmata,

is rapidly transferred into the vacuole to maintain the concentration gradient between phloem and parenchyma cells. During the final phases, the expression of *PpSUT4* decreases allowing the accumulation of high level of sucrose into the vacuole.

In order to unravel the possible role of each sucrose transporter isoform during peach fruit development, a tissue-specific study of transcript in specific mesocarp cell types, namely phloem and parenchyma cells, by LMPC was adopted. As expected from transcription analysis of the mesocarp tissue as a whole, *PpSUT1* appears almost undetectable in both cell types either at SI and SII stage of development.

These two approaches allowed to attribute a function to PpSUT2. In fact, *PpSUT2* is mainly localized in phloem cells, in particular, in specific cells of phloem bundles. Being sieve element highly modified cells, and lacking of nucleus and other organelles, the transcription likely occurs in the companion cells. Likewise, in Arabidopsis, the GUS expression of *AtSUC2*, *AtSUT2*, and *AtSUT4*, allowed the localization of the transcripts in companion cells of minor veins in leaves (Schulze *et al*., 2003). In addition, SUT2 proteins immunolocalized to sieve elements in *Arabidopsis*, *Plantago major* and Solanaceae species (Barker *et al*., 2000; Barth *et al*., 2003; Meyer *et al*., 2004). A role in sucrose sensing and signaling has been attributed to members of SUT2 subfamily (Barker *et al*., 2000). Taken together, these information suggest that PpSUT2 could function in the perception of carbohydrates availability and it may be postulated that PpSUT2, transcribed in companion cells, is transferred to sieve element, where it performs its activity.

On the contrary, PpSUT4 seems to play a role primarily in the distribution of sucrose in parenchyma cells. However, *in situ* hybridization showed a localization of *PpSUT4* transcript also in specific cells of phloem bundles, as *PpSUT2*. Conversely to PpSUT2, it may be postulated that, after translation, PpSUT4 localizes and functions in the companion cells

# 3. Chapter 2: sucrose distribution into peach seed

## 3.1. Introduction

### 3.1.1. Seed development: morphogenesis phase

In most tree species, a successful completion of pollination and fertilization of the ovule, followed by seed formation, is the prerequisite for fruit set and development. The processes of seed and fruit development which are intimately connected and synchronized, are controlled by phytohormones. Both pollination and seed-derived signals are required for fruit initiation and subsequent development. Fruit growth and shape are known to be modified by differences in seed number. In many species, including pepper, fruit size and fruit set have been reported to be positively correlated with seed number (Marcelis and Baan Hofman-Eijer, 1997). In some species, it can be observed the phenomenon of parthenocarpy, i.e. the growth of the ovary into a seedless fruit in the absence of pollination and fertilization (Gorguet *et al*., 2005). Fertilization-independent fruit set can occur either naturally (genetic parthenocarpy) or by induction via exogenous application of phytohormones, such as auxin and gibberellins (GAs), to the flower (Gustafson, 1936; Coombe, 1960).

A typical seed consists of three different tissues of distinct origin: two zygotic tissues, embryo and endosperm, and a maternal tissue, the seed coat. In Angiosperms, seed develops immediately after double fertilization, determined by the release of two sperm nuclei into embryo sac (Higashiyama *et al*., 2001) (Figure 36).

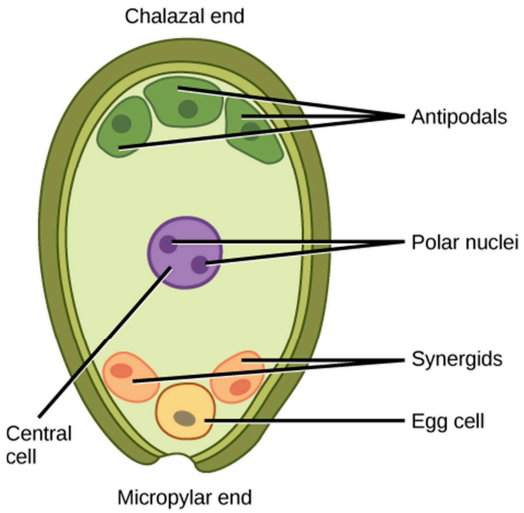


**Figure 36. Schematic representation of embryo sac in angiosperm showing the three antipodal cells, the two synergids, the central diploid cell, and the egg cell (from http://voer.edu.vn/module/reproductive-development-and-structure).**

The embryogenesis starts when the two sperm nuclei released from pollen tube fertilize the egg cell, which develops into the embryo (diploid), and the diploid central cell, that originates the endosperm (triploid). The function of the endosperm is to supply of nutrients the embryo during seed development (Baud *et al*., 2002).

After fertilization, embryo starts to growth until it fills the seed sac by intense mitotic activity (pre-storage phase) (Goldberg *et al*., 1994). The endosperm, which starts to divide immediately after fertilization, is then degraded to supply of nutrients the embryo (Brown *et al*., 1999). At the same time, maternal tissues start to change, and in particular the ovule integuments differentiate to form the protective seed coat, and the ovary develops into a fruit (Gehring *et al*., 2004; Baud *et al*., 2008).

The maturation phase, characterized by seed growth arrest, storage of several compounds, acquisition of desiccation tolerance, and entry into a dormancy phase (Harada, 1997) starts once embryo and endosperm have completed the morphogenesis (Wobus *et al*., 1999). The storage of reserve compounds is necessary to ensure the growth of seedling after germination, and usually consist of starch, triacylglycerols (TAGs) and specialized storage proteins (SSPs), the relative proportions of which vary greatly depending on the species considered. Storage compounds contribute up to 90% of the seed dry weight (Gutierrez *et al*., 2007).

The accumulation of reserves during the maturation phase can be conveniently divided into three distinct stages. Early maturing embryos display high starch concentration and start to accumulate storage proteins and lipids (Baud and Graham, 2006; Hills, 2004). During the second maturation phase, the starch levels decline, while processes of synthesis determine the high increase of fatty acids and proteins that lead to a rise in seed dry weight (Baud *et al*., 2002). Finally, late maturing embryos, characterized by accumulation of reserve within cotyledons, become metabolically quiescent and tolerant to desiccation. The water content decreases, the synthesis of storage compound ends, while oligosaccharides, such as raffinose, stachyose, sucrose and trealose, are accumulated and contribute to the acquisition of tolerance desiccation (Bailly *et al*., 2001; Hoekstra *et al*., 2001).

### 3.1.2. Sucrose metabolism in seed

Sugar content in seeds plays an important function during all phase of development. In fact, the imported sucrose serves either as a source of energy for cell growth and expansion, and as a substrate for storage reserves. Once unloaded in the filial tissue, it can be hydrolyzed outside the

embryo by a cell wall-invertase, then uptaken as hexoses, or directly loaded and cleaved within embryo by the activity of sucrose synthase (Figure 37). In general, in sink tissues, the invertase pathway is directed towards growth and cell expansion, whereas the sucrose synthase pathway is associated with storage product biosynthesis (Quick and Schaffer, 1996). In peach seed, high expression levels of *PpSUS1* have been detected during the late development stages (Falchi *et al*., 2013). Mutant lacking invertase activity shows inhibition in the early stages of seed development (Cheng *et al*., 1996). In legumes, the activity of a cell wall-bound invertase is restricted to early development and is responsible for the hydrolysis of sucrose after unloading from the seed coat. It produces an environment rich in hexoses stimulating cell division and expansion in embryo (Weber *et al*., 1996; Borisjuk *et al*., 1998). When the embryo expands and endosperm starts to be absorbed, invertase activity disappears. As a result, the hexoses concentration drops, and sucrose becomes the main endosperm sugar, concomitantly with the beginning of storage phase. The role of sucrose as a stimulator of sugar accumulation has been extensively reported. Treatment of young cotyledons of pea and *Vicia faba* favours storage product accumulation (Borisjuk *et al*., 2002). Seed tissues involved in storage have low level of acid invertase activity, but high levels of sucrose-synthase activity, a main route for entry of sucrose into cellular metabolism. In fact, reduction of its activity decreases the availability of assimilate for storage (Craig *et al.*, 1999). Sucrose synthase has frequently been cited as a marker for sink strength. The reaction of this enzyme is readily reversible, but it becomes irreversible by rapid removal of the cleavage products. The transcription of the gene for sucrose synthase is induced by sucrose (Weschke *et al*., 2003).

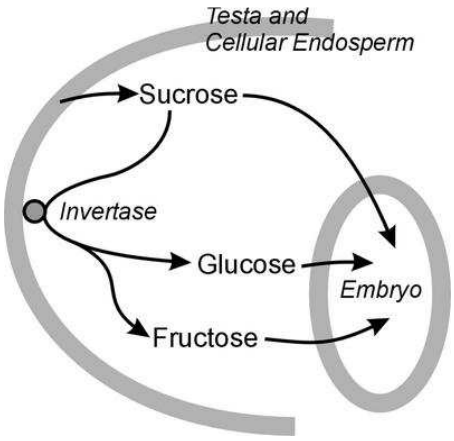


**Figure 37. Schematic view of sugar movements within a developing seed of oilseed rape (from Lionel *et al*., 2003)**

### 3.1.3. Seed nutrient supply

Seed is classified as sink organ, since it is totally dependent on nutrients supplied by the parent plant for its growth and development. Nutrients are transported toward seed tissues through the phloem transport, a dynamic process highly regulated. The amount of photoassimilates imported the final size, fundamental trait in seed crop production (Patrick and Offler, 2001). The transport of nutrients within the seed has been extensively investigated. Phloem unloading and post-phloem transport have been studied in the caryopses of wheat (Fisher and Cash-Clark, 2000), rice (Krishnan and Dayanandan, 2003), and in grain legumes (Patrick and Offler, 2001). It was found that the maternal and filial tissues are symplasmically isolated, whereas the vascular tissue distributed throughout tegument is connected to the surrounding cells by functional plasmodesmata. Thus, photoassimilates transported into the seed are released from the vascular strands, through a process driven by passive diffusion, and distributed throughout tissue that constitute the tegument. Photoassimilates have to pass apoplasmic space to reach endosperm and embryo tissue, taking advantage of the activity of transporters (Patrick and Offler, 2001).

### 3.1.4. Sucrose transporters in seed

The activity of sucrose transporters in seed is necessary to ensure the transfer of sucrose from maternal to filial generation, as they are symplasmically isolated, and for its transport from the apoplast into the embryo (Wolswinkel, 1992; Matsukura *et al*., 2000). Several genes encoding sucrose transporters have been isolated in developing seeds, such as *HvSUT1* and *HvSUT2* from barley (Weschke *et al*., 2000). In fava bean, after release in the apoplast, sucrose is differently uptaken according to development phase (Weber *et al*., 1997). Hexoses can be produced by extracellular hydrolysis, during the pre-storage phase, when the tissue is mitotically active. Concomitantly, both monosaccharide transporter gene (*VfSTP1*) and a cell-wall invertase gene are highly expressed. Otherwise, sucrose transport can be mediated by the activity of sucrose transporter (*VfSUT1*), highly expressed during the storage phase (Weber *et al*., 1997). The seed-specific overexpression of *StSUT1* in pea leads to an increase of sucrose uptake and growth rate in developing cotyledons (Roche *et al*., 2002). Mutation of *Atsuc5* gene leads to a strong alteration of fatty acid compositions in the endosperm of Arabidopsis seed, with a reduction of oleic and icosenic acid, and an increase of palmitic, lonolic and linolenic. Since fatty acid biosynthesis depends on the biotinylation, and AtSUC5 mediate the transport of biotin in addition to sucrose (Ludwig *et al*., 2000), it is not clear whether the phenotype depends on a different supply of the

cofactor or carbon source. However, the defect in *Atsuc5* gene is associated with a delay in embryo development (Baud *et al*., 2005).

### 3.1.5. Development of peach seed

The embryogenesis is crucial for the development of fruit in *Prunus persica*. Seed development is characterized by well-define phases (Ognjanov *et al*., 1995; Figure 38). It starts with a fast development of the nucellus, the central region of the ovule in which the embryo grows, that occurs from the end of blooming period. Initially, embryo derives carbohydrates, nitrogenous compounds, and mineral elements from the nucellus in which is imbedded (Bassi and Ryugo, 1990). Concomitantly, endosperm develops, starting immediately after fertilization, with a rapid nuclear division without cytokinesis accompanied by embryo sac expansion. The endosperm cellularization usually starts 35-40 days after anthesis, and ends in about a week. Then, endosperm grows very fast, concurrently with the nucellus re-absorption, and lasts until the beginning of endocarp lignification, when the seed reaches its final size. At the end of pit hardening, seed volume is mainly made by endosperm, and the embryo is at the heart stage. Thereafter, the embryo starts to increase in volume with the development of cotyledons, while endosperm is progressively hydrolized providing precursor and space for embryo growth. Hence, in peach, endosperm contributes very little to embryo storage materials, as in almond, in which metabolites, stored in the testa, nucellus and endosperm during the first growth stage, contribute only about 10% of the storage material finally accumulated by the embryo (Hawker and Buttrose, 1980).

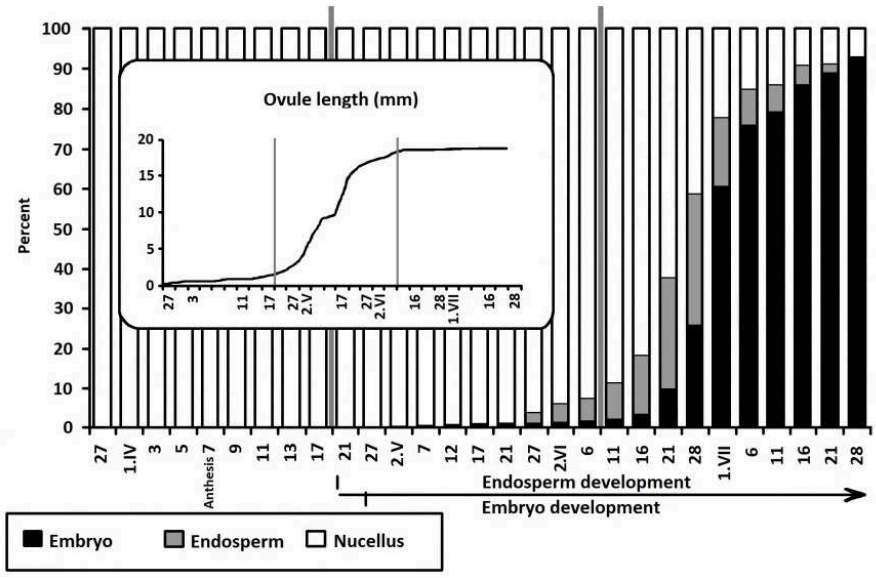


**Figure 38. Development of nucellus (white), endosperm (grey) and embryo (black) of Redhaven peach, expressed as proportion in the ovule, during development (from one week before anthesis to ripening). Top graph represent seed growth expressed as ovule length (mm) (from Ognjanov *et al*., 1995).**

The analysis of seed development in peach cultivar with different maturation time highlighted that fertilization is important for fruit set, mostly during the earlier phase, but the relationships between fruit development and embryogenesis become less strict in later phases. In fact, in early ripening genotypes, fruit mature when the development of seed is still in progress, and the seed is unable to complete the maturation phase, even if fruit can reaches normal size and maturation state (Bonghi *et al*., 2011).

## 3.2. Methods

Material was collected from peach plants of cultivar Redhaven (Prunus persica (L.) Batsch) cultivated in the Experimental Farm "A. Servadei" of Udine University in north-eastern Italy (46.01N, 13.13E). Seed growth was monitored weekly from about 50 dafb until harvest by measuring both fresh and dry weight of a pool of seed collected from ten different plants. Fresh weight is the weight of the seed just collected. The dry weight was estimated from the samples after oven drying. For each sampling, maternal tissues (tegument) were separated from filial tissues (endosperm and embryo) and stores at -80°C for subsequent analysis. Moreover, seed pieces of about 4 $mm^2$ in size, representative of different development stages, were embedded for subsequent analysis.

Embedding, methylene blue staining, analysis of phloem transport with carboxyfluorescein , RNA extraction, DNase treatment, cDNA synthesis, gene expression analysis by Real-Time PCR and *in situ* hybridization were performed as previously described in chapter 1.

## 3.3. Results

### 3.3.1. Seed growth curve

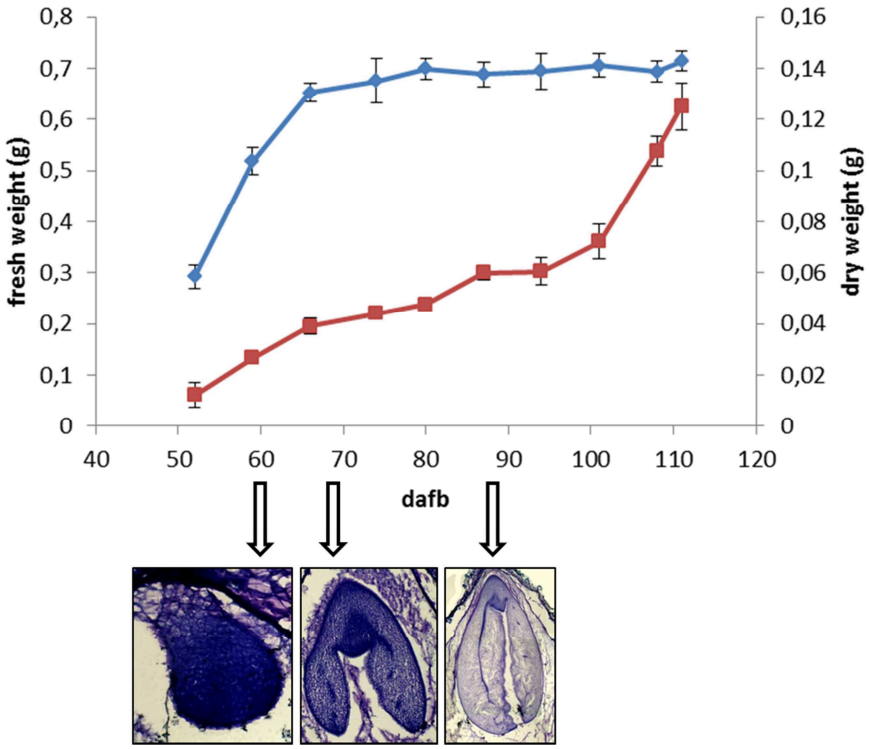


**Figure 39. Growth curve of peach seed (cultivar Redhaven), expressed as fresh weight (blue line) and dry weight (red line), during development. Pictures below show embryonic stage at about 60, 70 and 90 dafb.**

The growth of peach seed (cultivar Redhaven) was followed measuring both fresh and dry weight over time, starting from 52 dafb to 111 dafb (Figure 39). In terms of fresh weight, the growth pattern presented a single rapid increase with maximum value reached at about 70 dafb, after which remained constant throughout development. Differently, when expressed as dry weight, a continuous increase was observed in all phase of development. In detail, a rapid growth rate was detected in the early (from about 50 dafb to 70 dafb), and late (from about 100 dafb to 111 dafb) development stages, while the middle period was characterized by a slowdown of growth.

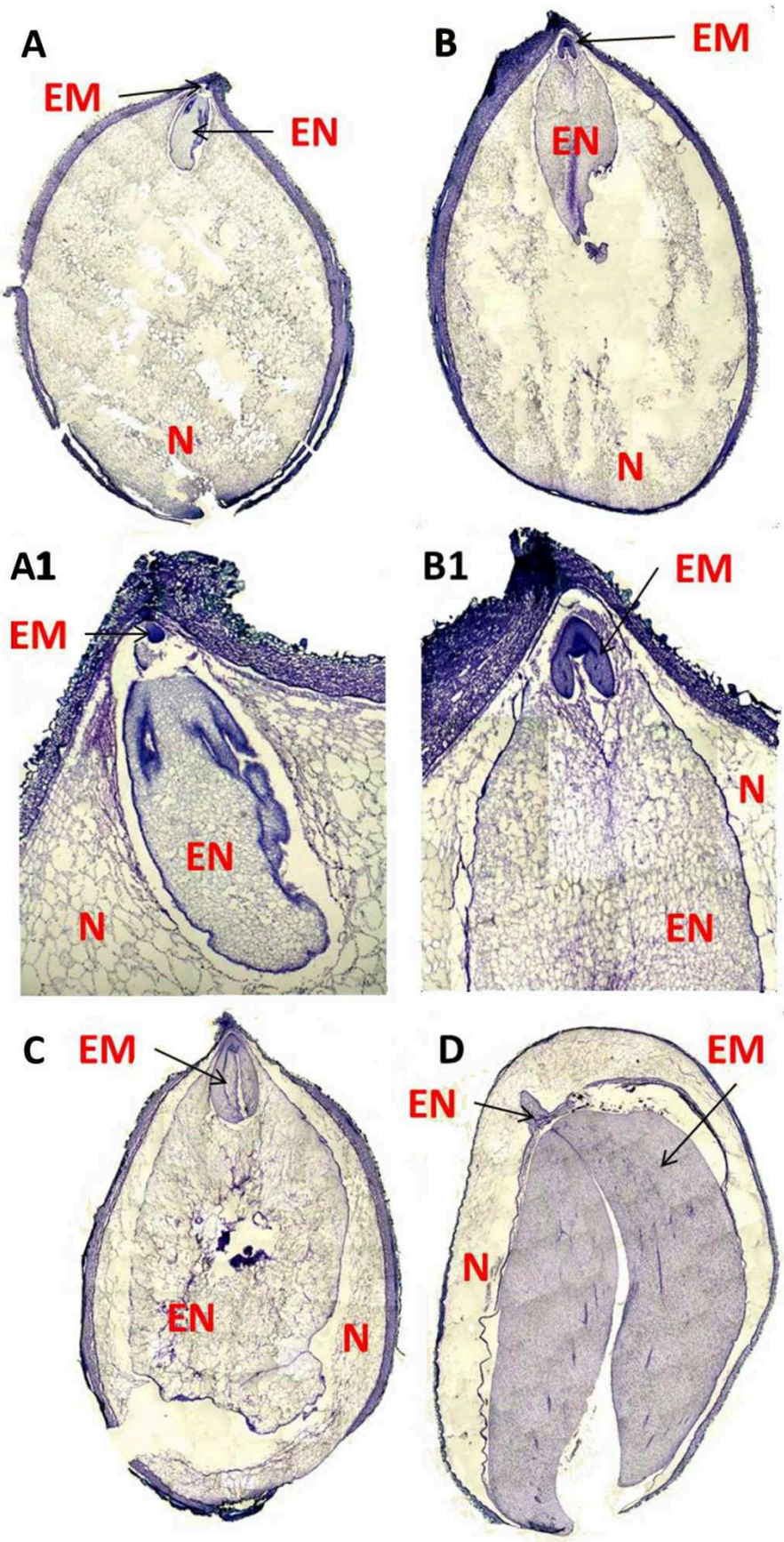


**Figure 40. Sections of peach seed at 60 dafb (A), 70 dafb (B), 90 dafb (C) and 105 dafb (D). A1 and B1 magnification of A and B, respectively. N: nucellus; EN: endosperm; EM: embryo.**

Inclusion and staining of peach seed allowed to identify the embryonic phase at different seed growth stages (pictures in Figure 39). At about 60 dafb, concurrently to the rapid growth of seed fresh weight, embryo was still at globular phase, while at approximately 70 dafb, when seed has reached its final dimension, started to differentiate the two cotyledons. At 90 dafb, cotyledons were more expanded. Proportion and develop of different tissues constituting the seed, i.d nucellus, endosperm and embryo, are shown in Figure 40. At about 60 dafb (Figure 40A), most of the seed consisted of nucellus, while endosperm was still underdeveloped and embryo, at globular phase, almost undetectable (Figure 40A1). After about ten days (Figure 40B), endosperm occupied more seed volume and embryo showed the two protrusions constituting the two cotyledons (Figure 40B1). At approximately 90 dafb (Figure 40C), phase preceding the second rapid growth in dry weight, the nucellus was almost completely absorbed, while endosperm occupied most of seed volume. The cotyledons were more developed and distinguishable. Finally, mature seed (Figure 40D) was mainly constituted by cotyledons, while endosperm was present as a thin layer closer to the embryo. Nucellus was still present.

### 3.3.2. Phloem strands distribution in peach seed

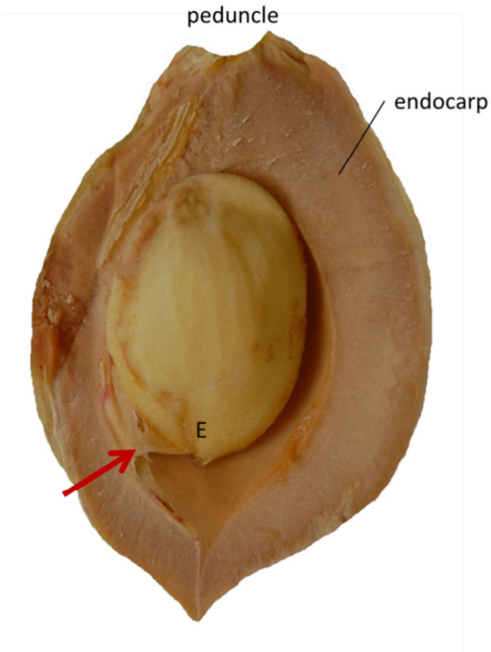


**Figure 41. Image of peach seed inside the endocarp. Arrow highlights the vascular bundles supplying the seed. E: region where embryo starts to develop.**

The peach seed is encased into a lignified endocarp (Figure 41). Several observation, by optical and stero microscope, of the structures allowed to identify vascular bundles, departing from the pedicel toward the seed. The attachment site of the vascular bundle is closer to the region where

embryo develops. Staining of young seed (about 25 dafb) sections allowed to analyze the distribution of vascular strands inside the reproductive organ (Figure 42). During this phase, the embryo is still not visible, and the main tissue filling the seed is the nucellus. It was observed that several vascular strands distributed all around the tegument.

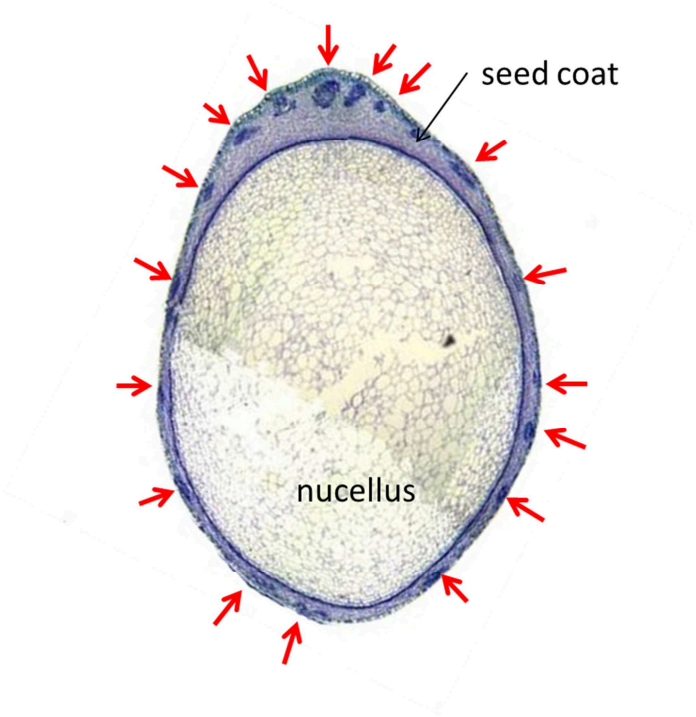


**Figure 42. Staining of seed section during early phase of development (about 25 dafb). Red arrows indicate vascular strands distributed throughout tegument.**

### 3.3.3. Analysis of mechanism of phloem unloading in seed by carboxyfluorescein

The study of phloem transport in peach young seed was carried out by the analysis of carboxyfluorescein (CF) distribution. To introduce the fluorescent tracer into the seed, peach shoots carrying one fruit and several leaves was fed with an aqueous solution supplemented with CF. As shown in Figure 43, the fluorescent tracer was able to move along the axis until reaching the reproductive organ. The image is a snapshot of the main vascular strand embedded in the endocarp, that at 25 dafb, is not lignified. The signal was very bright due to vascular strands abundance.

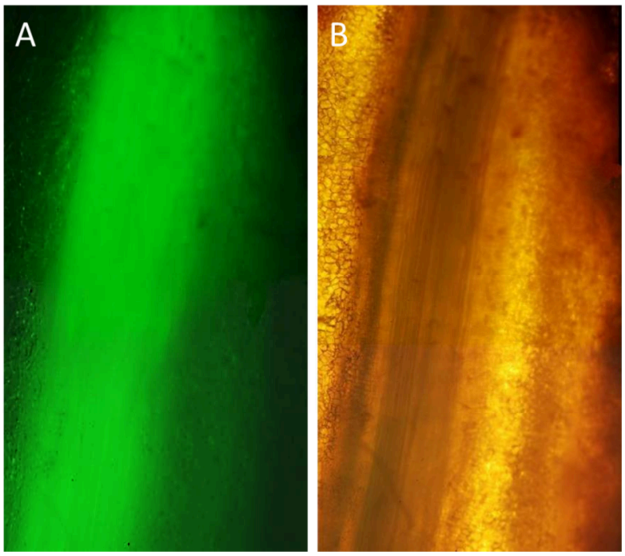


**Figure 43. Carboxyfluorescein distribution in the main vascular bundle connecting the peduncle to the seed. A: image taken under fluorescent light; B: image taken under transmission light.**

The analysis of CF distribution in seed is shown in Figure 44. The phloem tracer distributed uniformly throughout seed coat. Taken together, these results allowed two considerations: firstly, the phloem strands located in the tegument are symplasmically connected with adjacent cells by functional plasmodesmata, as CF diffused homogeneously from the phloem tissue to all surrounding cells; secondly, the maternal tissues are isolated from filial tissues, as CF did not spread inside the seed.

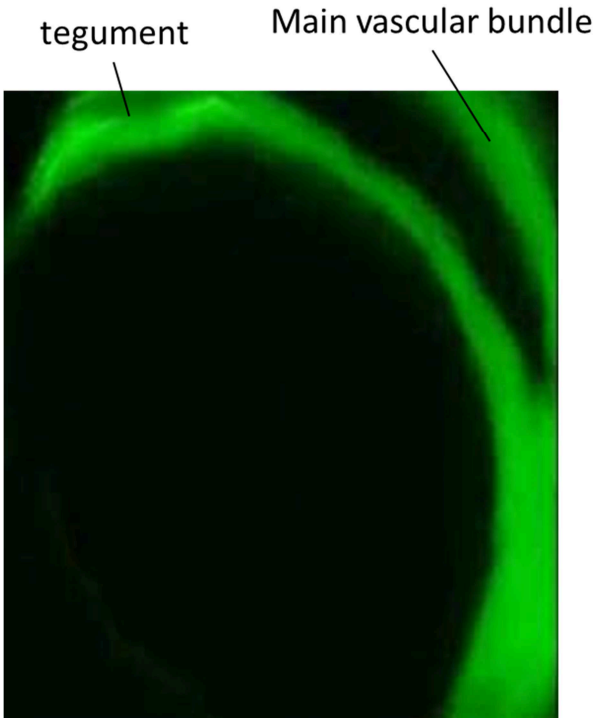


**Figure 44. CF distribution in peach seed.**

### 3.3.4. Analysis of PpSUT genes expression

**Real-Time PCR**

To acquire information about the putative functions of different members within SUT family in the distribution of sucrose, during development of peach seed, the expression level of their transcripts were evaluated by Real Time PCR, either in maternal (tegument) and filial (embryo and endosperm) tissues. The study showed that all genes investigated were expressed in all stages of development, with different trends and levels.

In the case of tegument, *PpSUT4* was the most abundant transcript and presented a modulated pattern during development (Figure 45). In detail, it showed low expression in early phases, rapidly increased reaching a maximum at 57 dafb, followed by a constant decreasing until 92 dafb, and a marked peak at 106 dafb. On the contrary, both *PpSUT1* and *PpSUT2* exhibited low expression level throughout tegument development, with slight differences. The *PpSUT1* (Figure 46) transcript levels were fairly constants, with minor increasing in the earlier (43 dafb and 57 dafb) and later (92 dafb and 106dafb) phases of growth. *PpSUT2* (Figure 47) displayed expression levels comparable to those showed for *PpSUT1*, but peaked at 57 dafb and 71 dafb, with minor increase at 106 dafb.

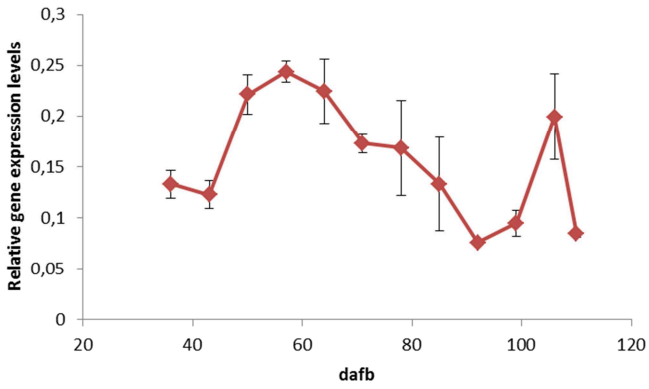


**Figure 45. Relative abundance of PpSUT4 transcripts detected by Real-time PCR in peach tegument of cultivar Redhaven during development. The bars represent standard deviation calculated from the mean of three replicates.**

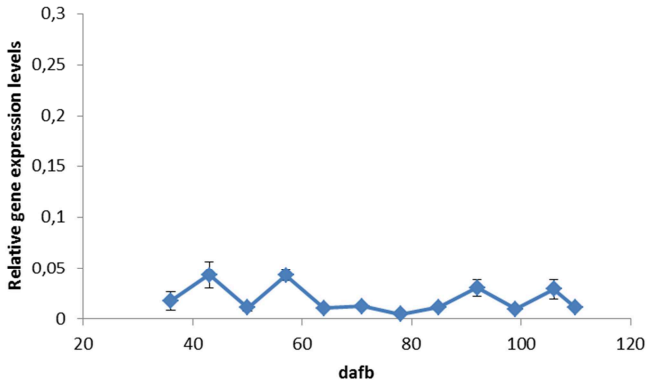


**Figure 46. Relative abundance of *PpSUT1* transcripts detected by Real-time PCR in peach tegument of cultivar Redhaven during development. The bars represent standard deviation calculated from the mean of three replicates.**

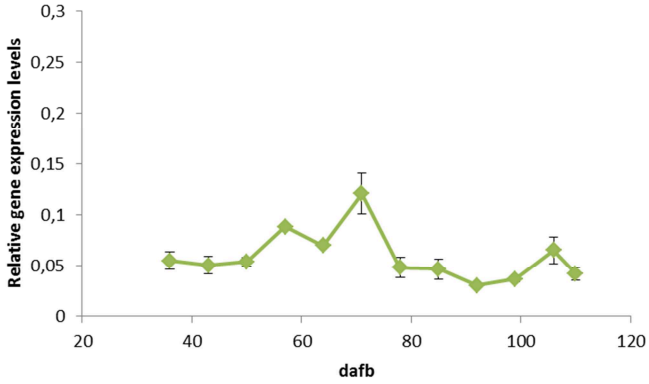


**Figure 47. Relative abundance of *PpSUT2* transcripts detected by Real-time PCR in peach tegument of cultivar Redhaven during development. The bars represent standard deviation calculated from the mean of three replicates.**

Differently, the quantification of PpSUT transcript in filial tissues displayed variations in the abundance and pattern, as compared with maternal tissues. All genes exhibited highly modulated trends. PpSUT1 (Figure 48), the most abundant transcripts at least in the early phase of development, picked at 57 dafb, then constantly decreased until the end of seed development, with the exception of a transient increase at 85 dafb. PpSUT2 (Figure 49) showed a more uniform profile. In fact, it displayed moderate rises at 43 dafb, and 106 dafb, and an additional increase, that takes place at 71 dafb. PpSUT4 (Figure 50) exhibited an interesting trend with a first constant rise until 57 dafb, followed by almost unchanged relative transcript levels until 92 dafb, and an up-regulation at 106 dafb.

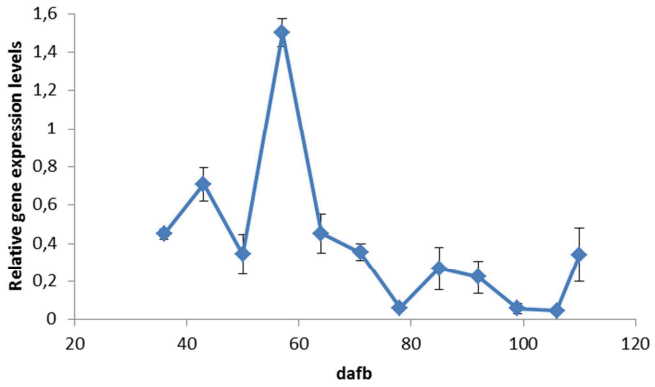


**Figure 48. Relative abundance of *PpSUT1* transcripts detected by Real-time PCR in peach filial tissues (embryo and endosperm) of cultivar Redhaven during development. The bars represent standard deviation calculated from the mean of three replicates.**

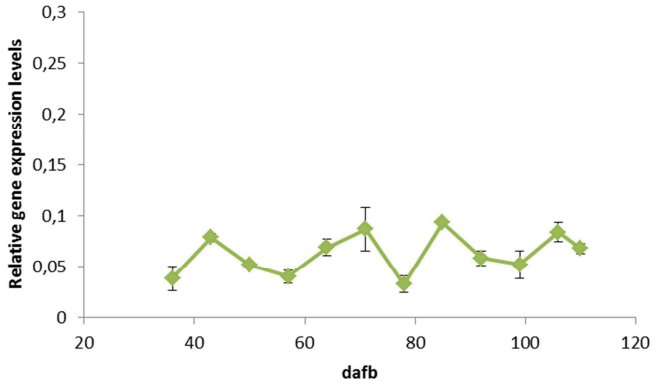


**Figure 49. Relative abundance of *PpSUT2* transcripts detected by Real-time PCR in peach (filial tissues) embryo and endosperm of cultivar Redhaven during development. The bars represent standard deviation calculated from the mean of three replicates.**

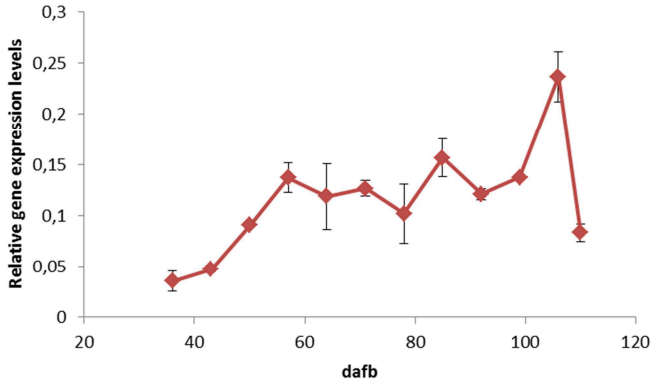


**Figure 50. Relative abundance of *PpSUT4* transcripts detected by Real-time PCR in peach (filial tissues) embryo and endosperm of cultivar Redhaven during development. The bars represent standard deviation calculated from the mean of three replicates.**

### *In situ* hybridization

To analyze in greater detail the expression of the three *PpSUT* genes, experiments of *in situ* hybridization were carried out in seed tissues, at two different growth stages (50 and 85 dafb). For each gene, two different RNA probes labeled with digoxigenin were designed in antisense and sense direction, the latter with the aim of ensure the specificity of the hybridization. The successful interaction between antisense probe and mRNA was observed through a colorimetric assay. The expression of the three *PpSUT* genes was evaluated in serial sections of the same sample in two different phase of development (50 and 85 dafb).

In young seed, the three anti-sense probes, labeled differently sequential tissue sections (Figure 51). The better signal was detected for *PpSUT1*, which labeled markedly the phloem tissue, as shown at high magnification (Figure 52). However, the comparison between tissues treated with anti-sense and sense probes allowed to identified other sites of hybridization, namely cells forming the nucellus.

Differently, in peach mature seed (Figure 53, Figure 54) the labeling of tegument became less evident for all three genes. In detail, *PpSUT1* transcripts appeared almost absent, whereas a light signal was detected in sections treated with *PpSUT2* and *PpSUT4*. Concerning *PpSUT2*, a good staining of phloem cells has been evidenced in endosperm tissue; interestingly, *PpSUT4* transcripts have been detected also in cotyledonous.

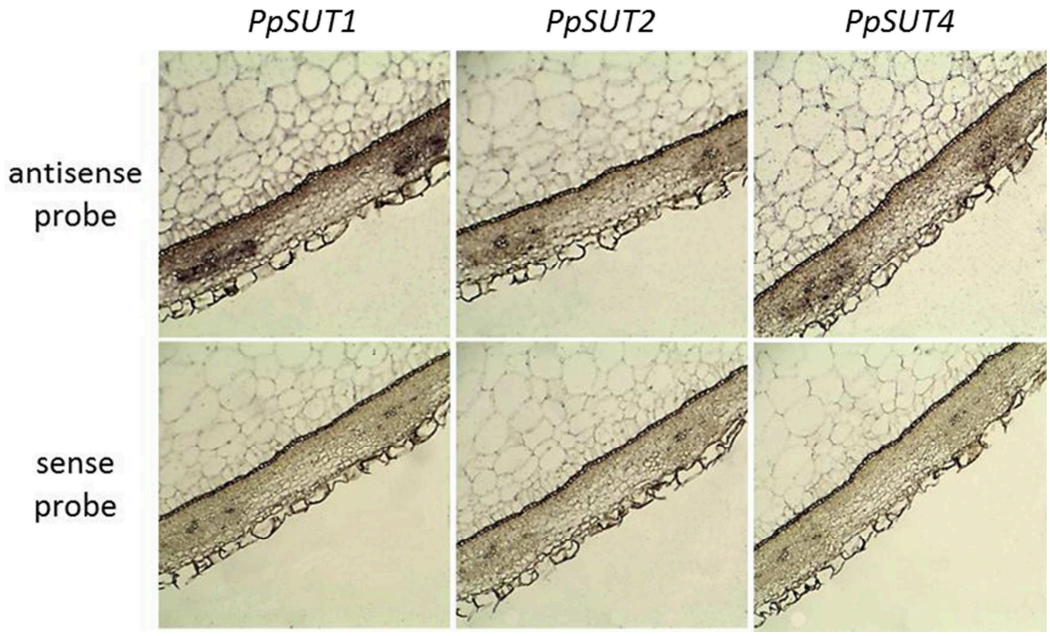


**Figure 51. Gene expression localization of *PpSUT1*, *PpSUT2* and *PpSUT4* in serial sections of seed tissue during early phase of development (50dafb) by means of *in situ* hybridization.**

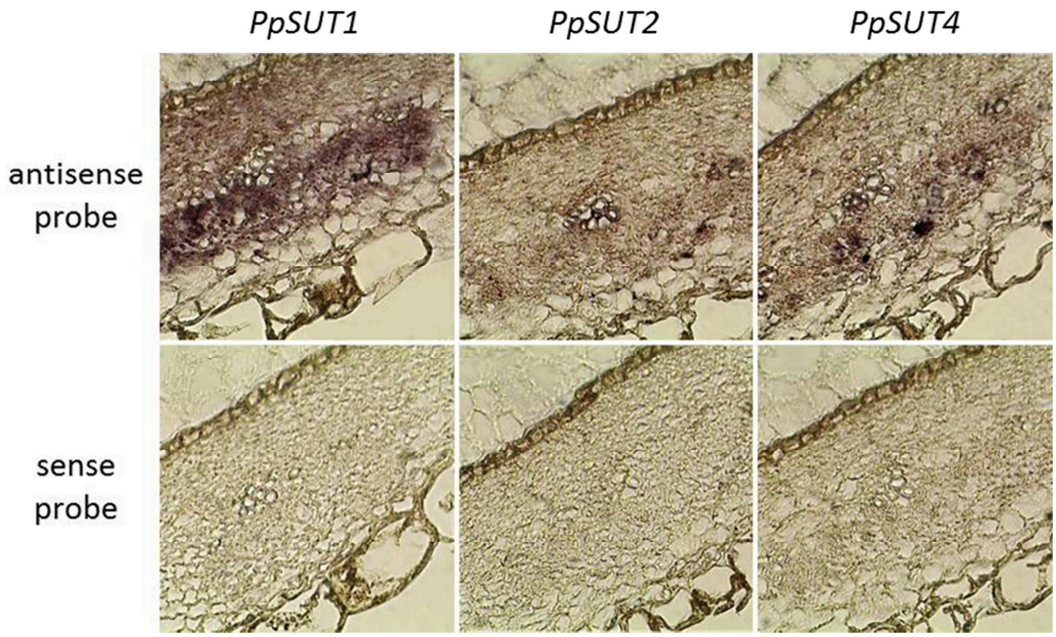


**Figure 52. High magnification of phloem strand located in the tegument of young seed (50dafb) treated with *PpSUT1*, *PpSUT2* and *PpSUT4* probes.**

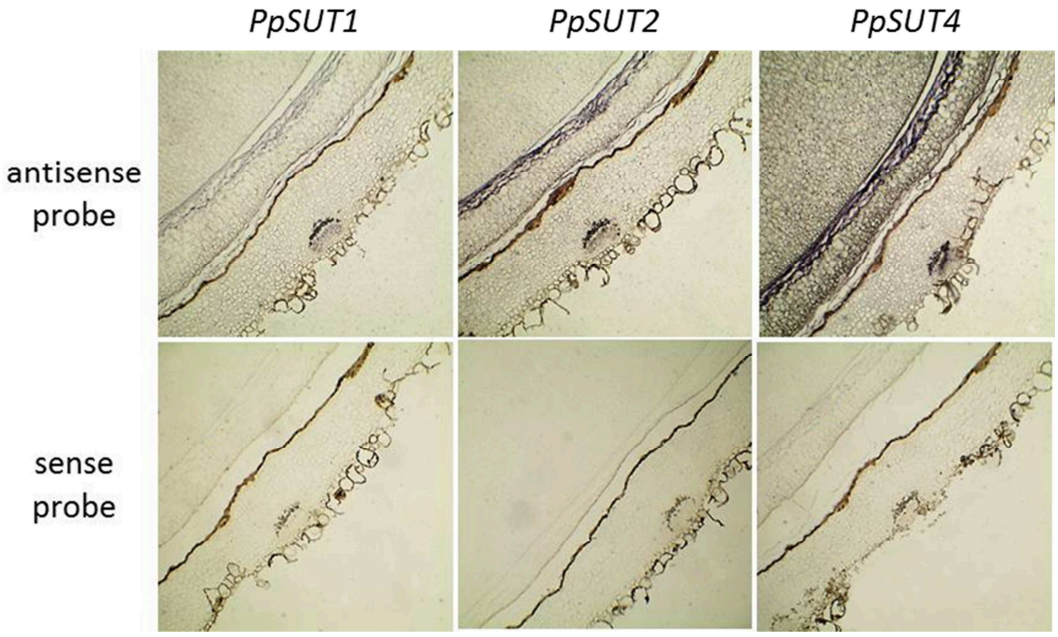


**Figure 53. Gene expression localization of *PpSUT1*, *PpSUT2* and *PpSUT4* in serial sections of seed tissue during late phase of development (85 dafb) by means of *in situ* hybridization.**

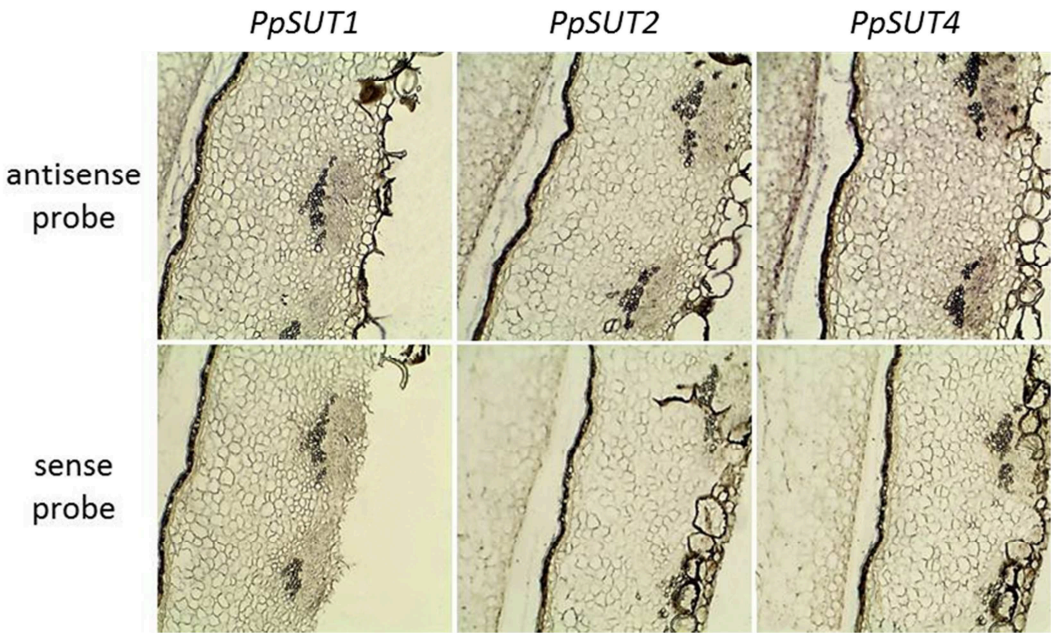


**Figure 54. High magnification of phloem strand located in the tegument of mature seed (85 dafb) treated with *PpSUT1*, *PpSUT2* and *PpSUT4* probes.**

## 3.4. Discussion

Seed development is a crucial process in the lifecycle of higher plants, providing the link between two distinct sporophytic generations, and, thus, the maintenance of the species. Moreover, seed plays an additional essential function, being necessary for fruit set. Indeed, although parthenocarpic fruits can develop, prolonged fruit development usually relies on the constant presence of developing seeds. The number of fertilized ovules in a fruit is correlated with both the initial cell division rate and the final size of the fruit (Marcelis and Baan Hofman-Eijer, 1997). What is more, fruits with an uneven distribution of seeds are often lopsided. Therefore, a successful fertilization has an important impact on fruit crop production.

In *Prunus persica*, fruit development is strictly connected to embryogenesis. Several efforts to stimulate parthenocarpic fruit development by hormone application have been unsuccessful. In addition, seed anomaly at the early stages of development leads to abortion and fruitlet abscission (Stutte and Gage, 1990). Generally, seed and pericarp grow synchronously, showing specific phases during development, and probably they are in competition with each other for assimilates transported through phloem sap. The seed's ability to unload carbohydrates from the phloem defines its sink strength, and may be important to prevent the loss of sugar along the transport pathway, before reaching its tissues. Peach seed, as the pericarp, exhibits a modulated pattern of growth. The most critical phase is characterized by a slowdown in growth rate, concomitantly with an intense mitotic activity of the embryo (Bonghi *et al.*, 2011). The seed is a complex organ, as constitute of tissue, with different origins, exhibiting distinct metabolism and needs. The photoassimilates reaching the seed are firstly used as a source of energy for cell division and metabolism, but in part employed as a storage reserve. Moreover, different tissues participate to store reserve in distinct development phase, i.e. earlier nucellus, and endosperm, and later cotyledons.

There is a unique route that allowed assimilated to reach seed tissues, that is through a main vascular bundles connecting pedicel to seed. Analysis of CF distribution allowed to make two observations: phloem cells are connected with surrounding cells, and the maternal tissues are apoplasmically isolated from filial tissues, as already observed in other plant system (Patrick and Offler, 2001). Therefore, the present work permitted to hypothesize that the sugars transported into seed by the phloem system are released from SE/CC complex and pass from one cell to another through plasmodesmatal connection, spreading in all cells of the tegument. However,

when carbohydrates reach the internal layer of tegument, encounter an interruption of simplasmic continuity. To reach endosperm and embryo, sugars, mainly sucrose, have to be transported across the plasma membrane of the cells of tegument and filial tissues.

To gain information concerning the involvement of sucrose transporters in sink seed, the expression of three putative genes encoding sucrose transporters were analyzed in two distinct tissues, maternal and filial. Surprisingly, all three genes were expressed in both tissue types. *PpSUT4* was the most abundant transcript detected in maternal tissue and more modulated. In other plant species members of SUT4 subfamily have been localized to the tonoplast (Chincinska *et al*., 2013), and their involvement in the regulation of sucrose efflux from the vacuole has been hypothesized (Christopher *et al*., 2012).

With this regard, it can be postulated a role for PpSUT4 in the regulation of sucrose concentration in the cytosol of tegument cells. During the first phase of growth, seed structures growth very rapidly, whereas endosperm cells start dividing, and embryo grows slowly. In the tegument, through a symplasmic mechanism of phloem unloading, carbohydrates move from SE/CC complex to surrounding parenchyma cells. To maintain the concentration gradient that drives passive diffusion, most of unloaded sucrose is rapidly imported into the vacuole of tegument cells. It may be hypothesize the presence of an $H^+$/antiporter to efficiently transfer of disaccharide into storage organelle. The expression of *PpSUT4* would be necessary to regulate the release of sucrose into the cytosol, as energy source for tegument growth. As seed reaches its final dimension and tegument terminates its growth (at the end of first phase of seed growth), *PpSUT4* is down regulated because sucrose is no longer necessary as substrate. Thus, most part of sugars imported into the seed can reach the filial tissues and sustain their growth.

*PPSUT1* and *PpSUT2* are expressed in lesser extent, but in situ hybridization revealed that *PpSUT1* is primarily localized in phloem tissue in the early stages of development. As all members of SUT1 clade have been address to the plasma membrane, and have been proposed as a marker of companion cells of the phloem (Ivashikina *et al*., 2003; Deeken *et al*., 2008), *PpSUT1* might be localized to the plasma membrane of SE/CC complex and play a role in the exchange of sucrose between parenchyma and phloem cells. Further, its function might be linked to the retrieval of sucrose, during long-distance transport into SE/CC complex, as all sucrose transporters characterized to date are $H^+$/symporters (Kühn and Grof, 2010).

The expression analysis carried out in filial tissues exhibited a highly modulated expression over time, that might be linked to different metabolic conditions of the three tissues (endosperm,

embryo, and in earlier phase, also nucellus). They showed an up-regulation in distinct phases of seed development. A high increase of *PpSUT* transcript abundance was clearly observed at 55 dafb, in particular for *PpSUT1*, phase characterized by ah high seed growth, and a rapid endosperm cell division. In this phase, embryo size is still extremely small (Ognjanov *et al*., 1995). In the endosperm, the presence of *PpSUT1* at the plasma membrane of endosperm cells allow the movement of the disaccharide into the temporary storage tissue. The high expression of *PpSUT1* was observed concomitantly with the rapid growth of seed and tegument enlargement. It is suggested that most sugars reaching the seed in the first phase of seed development are used to sustain growth of tegument. SUT1 proteins are characterized as high affinity transporters (Kühn and GRof, 2010), and hence are able to transport sucrose also when present in low concentration. Thus, the presence of high/affinity PpSUT1 at the plasma membrane of endosperm cells ensures the transport of sucrose into the temporary storage tissue, even if the amount reaching the filial tissue is low. The activity of PpSUT1 maintains low sucrose concentration into the apoplast and, consequently, the concentration gradient driving passive diffusion from maternal to filial tissue. It is assumed the presence of a sucrose carrier at the plasma membrane of the internal cell layer of the tegument allow the transport of disaccharide between the two tissues.

Moreover, the *in situ* hybridization in a seed during the second phase of growth, revealed a localization of *PpSUT2* and *PpSUT4* transcripts in the interface between endosperm and embryo. During this phase, embryo grows rapidly due to active cell division, and the reserves temporary stored into the endosperm are transferred to the embryo, consequently endosperm is degraded and reabsorbed (Ognjanov *et al*., 1995). The presence of *PpSUT* genes indicates an important re-distribution of sucrose between the two tissues. On the other hand, the detection of *SUT* genes in the endosperm transfer cells has already been described in barley (Weschke *et al*., 2000).

During the last phase of development, at the beginning of the second rapid increase of seed dry weight, when embryo has reached its final dimension and started to synthesize storage compound (Ognjanov *et al*., 1995), was observed an increase in the expression level of *PpSUT4*. This carrier may contribute to determine the release of sucrose stored within the vacuole to be utilized in the cytosol. It has been observed, in fact, that during the last phase of seed development the transcription of gene encoding sucrose synthase increases (Falchi *et al*., 2013).

In conclusion, this work is a preliminary study about the putative function of sucrose transporters in the distribution of sucrose into different tissue forming the seed. It has been shown that three genes encoding sucrose transporter are expressed in peach seed, and a different role during

distinct phases of development has been suggested for these proteins. Main functions might be attributed to PpSUT4 and PpSUT1. It can be hypothesized a role of PpSUT4 in the efflux of sucrose to sustain growth and development of tegument and to supply substrates to cotyledons cells during later phase of development, in order to allow the synthesis of storage compound. PpSUT1 may hold an important function in the retrieval of sucrose, transported from the inner layer of tegument to apoplast, into the endosperm cells to allow the accumulation of temporary stored compound. Finally, PpSUT proteins have an important function during the process of endosperm re-absorption by the embryo. Further experiments are needed to validate this hypothesis.

# 4. Chapter 3: Characterization of PpSUT proteins

## 4.1. Introduction

### 4.1.1. Role of sucrose transporters in the whole-plant carbohydrate partitioning

The whole-plant carbohydrate partitioning involves the distribution of photoassimilates produced in source tissue toward different sink organs. The movement of sugars is strictly dependent on sucrose transporters activity, particularly in the absence (or closure) of plasmodesmata channels that symplasmically connect sieve tubes elements with adjacent cells.

In higher plants, several genes encoding sucrose transporters have been identified. Members of this family diversify for expression patterns, substrate affinity, substrate specificity, and subcellular localization. These differences reflect distinct physiological functions in plants. One of the main is the loading of sucrose into phloem leaves. The antisense inhibition of SUT1 in potato has led to an accumulation of carbohydrates in leaves and a retard in plant growth. As a consequence of the phloem loading impediment, tuber yield was dramatically reduced (Riesmeier *et al*., 1994). SUT proteins are proposed to play other roles along phloem path. Indeed, during transport, sucrose is released to feed tissues flanking phloem tissue, but could be retrieved back into sieve elements by sucrose transporters in order to maintain the hydrostatic pressure necessary for the movement of phloem sap (Hafke *et al*., 2005). A great number of sucrose transporters have also been detected in sink organs as seed (Weber *et al*., 1997; Tegeder *et al.*, 1998), pollen (Lemoine *et al*., 1999; Stadler *et al*., 1999), and fruits (Peng *et al*., 2011), in which they may function in the uptake of disaccharide into the cells.

Several researches hypothesized a regulative role for SUT proteins in carbon allocation and in the plasticity of phloem transport, necessary to optimize plant growth and development. In fact, the sink demand of assimilate may rapidly change in response to different environmental conditions or internal factors, the regulation of sucrose transporter activity may be an effective and fast mechanism to adapt resource allocation between different sink organs (Liesche *et al*., 2011).

### 4.1.2. Sucrose transporters activity

In general, transporters are highly hydrophobic proteins that allow the movement of a compound across biological membrane. There are different types of transporters: some mediate the diffusion of specific molecules along their concentration gradient, others catalyze the accumulation of compounds in a given compartment by coupling the substrate transport with ions transport, usually $Na^+$ and $H^+$. The latter represents the secondary active transport that utilizes the free energy stored in the electrochemical ion gradients for the movement of a compound against its concentration (Stein, 1986). The electrochemical potential is generated by the activity of $H^+$-ATPase that hydrolyzes ATP to generate a $H^+$ concentration gradient through biological membrane (Morsomme and Boutry, 2000; Palmgren, 2001).

The first analysis concerning sucrose transporters in higher plants was carried out more than thirty years ago (Giaquinta, 1976). The first cDNA encoding sucrose transporters was identified from spinach by functional characterization in yeast (Riesmeier *et al*., 1992). The yeast complementation system is a very powerful technique for functional analysis of sucrose transporters (Frommer and Ninnemann, 1995). It is based on the use of a mutant *Saccharomyces cerevisiae* strain, called SUSY7. Usually a wild type yeast strain lacks a system to directly uptake sucrose from the medium. Thus it has evolved a system to hydrolyze the disaccharide into glucose and fructose outside the cells, through the secretion of an invertase. The monosaccharides produced by the sucrose cleavage are then transported inside cell by an hexose transporters system (Carlson, 1981). In SUSY7 strain the genes encoding extracellular invertase and maltose permease (which is also capable to transport sucrose) have been deleted to avoid the cleavage of sucrose outside yeast cell and to avoid the direct uptake of sucrose; at the same time, a cytoplasmic sucrose synthase gene from potato has been inserted to allow the metabolization of ingested sucrose. This mutant is unable to grow on medium containing sucrose as a sole carbon source, because unable to transport and cleavage the external sucrose into hexose. The phenotype is restored when the mutant is transformed with a yeast expression vector carrying a cDNA encoding a functional sucrose transporter (Riesmeier *et al*., 1992).

So far, all identified SUT proteins have been characterized as sucrose/$H^+$ symporters (Ayre, 2011). In fact, it has been shown that sucrose uptake can be strongly inhibited by uncouplers of proton gradients such as carbonyl cyanide m-chlorophenylhydrazone (CCCP) or dinitrophenol (DNP) (Riesmeier *et al*., 1992; Riesmeier *et al*., 1993; Sauer and Stolz, 1994; Knop *et al*., 2004). Moreover, the transport activity is inhibited by the presence of a non-penetrating sulfhydryl group modifier

such as p-chloromercuribenzene sulfonic acid (PCMBS) and diethylpyrocarbonate (Riesmeier *et al*., 1992; Riesmeier *et al*., 1993). The heterologous expression of SUT proteins in *Xenopus oocytes,* and subsequent measurement of current after sucrose application using two-electrode voltage clamp, revealed that the transport activity depends on pH medium, with a pH-optima established at acidic range. In addition, the transport activity displayed a Michelis-Menten type kinetic with a 1:1 $H^+$/sucrose stoichiometry (Boorer *et al*., 1996; Zhou *et al*., 1997). Some exceptions have been identified. Recently, the analysis of ZmSUT1 transport activity from mays (*Zea mays* L.) has revealed the ability to catalyze both sucrose efflux and influx (Carpaneto *et al*., 2005). Moreover, two transporters from *Pisum sativum* and *Phaseolus vulgaris*, with high sequence similarity to the SUT4 clade, have been characterized in yeast as sucrose facilitators (SUFs), since they mediate a bi-directional transport of sucrose, in a pH- and energy-independent manner (Zhou *et al*., 2007).
The analysis of sucrose uptake kinetic into different plant tissues allowed to identify two carrier group: one characterized by high affinity/low capacity, and the other by low affinity/high capacity (Delrot and Bonnemain, 1981).

### 4.1.3. Post translational regulation of sucrose transporters

Several authors suggested that different factors participate in the regulation of sucrose transporters activity including protein interaction, subcellular localization, and redox-regulation (Liesche *et al*., 2011).

**Oligomerization**

One efficient mechanism of sucrose transporters regulation depends on protein-protein interaction (Krügel and Kühn, 2013). For example, oligomerization, was described to play a role for many membrane proteins (Veenhof *et al*., 2001), such as the mammalian glucose uniport GLUT1, belonging to the major facilitator superfamily as yeast hexose and plant sucrose transporters. Biochemical studies provided evidence that GLUT1 forms tetramers, composed as dimers of dimers (Hebert and Carruthers, 1992; Zottola *et al*., 1995). The four homomeric subunits coexist in two conformational states in which the substrate binding site alternates outside and inside the membrane. The binding of substrate to one transport site changes the substrate affinity of the interacting partner in a cooperative manner (Hamill *et al*., 1999).
In the last few years several authors have demonstrated the capacity of plant sucrose transporters to form dimers. The first evidence has been detected in the plasma membrane fraction of sugar

beet leaves (*Beta vulgaris* L.) after separation on a size exclusion chromatography under nondenaturing conditions. A sucrose transporter activity has been identified in a high molecular mass complex, about 120 KDa. The denaturation of the 120 KDa complex, and the rejniection on a gel filtration column gave a peak of different molecular weight, i.e. 42 KDa (Li *et al*., 1991).

The co-localization of different sucrose transporters in the same cell type has led several authors to investigate the possibility of a homo- and heterodimer formation. For instance, SUT proteins from Solanaceae (potato and tomato), characterized by different kinetic of transport and substrate affinity, have been localized in the same sieve element (Barker *et al*., 2000; Weise *et al*., 2000; Reinders *et al*., 2002a), while in Arabidopsis a co-expression of *AtSUC2*, *AtSUT4* and *AtSUT2* was observed in companion cells (Schulze *et al*., 2003). The main experimental approach used to demonstrate this hypothesis was the split-ubiquitin system (SUS). Unlike this method, the classical yeast two-hybrid analysis cannot be applied to the study of membrane protein interaction, because it requires the movement of the interacting proteins to the nucleus for reporter gene expression. In the SUS, ubiquitin is utilized to study the interaction between membrane proteins. This conserved 76 amino acid peptide, involved in the protein degradation by the 26S proteasome, is splitted in two parts, the N-terminal (NUB, aa1-34) and the C-terminal (CUB, aa35-76) moiety, that have been subsequently modified. Wild type NUB has an isoleucine at position 13 that confer a high affinity to CUB. To avoid the spontaneous reassembling of the two parts, the NUB has been altered through the replacing of Ile-13 with a glycine (NUBG). In addition, a transcription factor, necessary for the expression of the reporter gene, is added to the CUB. Finally, these two halves are fused to the protein to be analyzed. When expressed in the same cell, and if the two proteins interact, the ubiquitin is reconstituted and can be recognized by ubiquitin specific protease. This cleaves after the carboxy terminus of ubiquitin, realizing the transcription factor that, in turn, enters the nucleus and activates the reporter genes (Stagljar *et al*., 1998). The split-ubiquitin system is suitable to study protein-protein interactions between different SUT proteins. In fact, the expression of two separated halves of sucrose transporters from *Solanaceae* can restore a functional protein at the yeast plasma membrane (Reinders et al., 2002b). Through this heterologous system, the capability of different SUTs from *Arabidopsis* and tomato (*Solanum lycopersicum*) to form homo- and heterooligomers has been demonstrated (Reinders *et al*., 2002a; Schulze et al., 2003). Moreover, heterodimers between StSUT1 and StSUT4 have been detected *in planta* by BiFC experiments, mainly in the endoplasmic reticulum (Krügel *et al*., 2012).

It has been reported that, for many plant membrane proteins, oligomerization is a prerequisite for endoplasmic reticulum export and/or plasma membrane targeting. An example are the aquaporins from maize that only in the dimeric form may be exported from the ER and targeted to the PM (Maurel, 2007). Additionally, it has been demonstrated that the homooligomerization of SWEETs protein in *Arabidopsis* is needed for transport function (Xuan *et al*., 2013). The protein complexes formation may also influence their activity, as observed in Solanaceae in which SUT1 activity appeared negatively affected by the co-expression of SUT2 protein in heterologous system (Reinders *et al*., 2002a).

**Phosphorylation/dephosphorylation**

A common posttranslational modifications regulating protein activity involves phosphorylation/dephosphorylation. This mechanism offers a dynamic way to regulate protein activity, subcellular localization, protein-protein interactions and stability (Johnson, 2009), and has been described for many proteins, as monosaccharide transporters in yeast (Carlson, 1998).

However, there are several evidences that this mechanism is also involved in the regulation of sucrose transporters. In fact, the treatment of sugar beet (*Beta vulgaris* L.) leaf discs with okadaic acid, a protein phosphatase inhibitor, has led to a reduction of sucrose uptake. These data support the hypothesis that phosphorylation inhibits the activity of sucrose transporters (Roblin *et al*., 1998). Similar results have been observed later by Ransom-Hodgkins *et al.* (2003). Different phosphorylation sites have been identified in AtSUT proteins from *Arabidopsis*. Phosphorproteomics analysis provided evidence for the phosphorylation of the AtSUC5 at the N-terminus (Nühse *et al*., 2004). Detailed investigation by mass spectrometry determined the exact position at the highly conserved serine 20 (amino acid identified in all Arabidopsis sucrose transporter, except AtSUC2), and at the threonine 393 (Niittyla *et al*., 2007). Moreover, it has been shown that AtSUC6 from *Arabidopsis* can interacts with a 14-3-3 protein, involved in the regulation of several processes by binding to phosphorylated proteins (Shin *et al*., 2011).

**Subcellular localization**

The subcellular localization of membrane proteins represents a crucial aspect for their functionality. As far as sucrose transporters are concerned, all members of SUT1 and SUT2 subfamily have been localized to the plasma membrane (Gottwald *et al*., 2000). In detail, SUT1

proteins have been demonstrated by immunolocalization to be present in the plasma membrane of sieve elements in tobacco, potato, and tomato (Kühn *et al*., 1997), but also in the plasma membrane of companion cells (Stadler *et al*., 1995), and others in both cell types (Knop *et al*., 2004). Similarly, SUT2 proteins have been localized to the plasma membrane of sieve elements in tomato, plantain, and Arabidopsis (AtSUC3) (Barker *et al*., 2000; Barth *et al*., 2003; Meyer *et al*., 2004). Differently, SUT4 members have been reported to be target either to the plasma membrane or to the tonoplast in different plant species (Chincinska *et al*., 2013). AtSUT4 has been localized in the plasma membrane both by functional characterization in yeast and by studies of co-localization with plasma membrane transporters (Weise *et al*., 2000; Schulze *et al*., 2003). Vacuole proteomics allowed the localization at tonoplast level (Endler *et al*., 2006), and chloroplast proteomics also to the chloroplast envelope (the unique report of sucrose transporters at the chloroplast membrane) (Rolland *et al*., 2003). LjSUT4 has been recognized in the plasma membrane when expressed in *Xenopus oocytes,* and in the tonoplast when expressed as GFP fusion protein in plant (Reinders *et al*., 2008). NtSUT4 has been localized to the plasma membrane by functional characterization in yeast, and plant by western blot analysis (Okubo-Kurihara *et al*., 2011; Chincinska *et al*., 2013), while the analysis by GFP fusion protein targeted the transporter to the vacuole (Okubo-Kurihara *et al*., 2011). In apple fruits, MdSUT1 has only been localized to the plasma membrane of both sieve elements/companion cells and storage parenchyma cells by immunogold labeling (Peng *et al*., 2011).

The recent study on the intracellular localization of a Zinc-Induced Facilitator-Like 1 (ZIFL1), a Major Facilitator Superfamily (MFS) transporter, suggested that the subcellular localization of membrane proteins may vary according to different splicing of mRNA. In fact, the ZIFL1 is expressed in two isoforms targeted toward two distinct membranes. The full-length protein localized to the tonoplast of root cells, while the truncated isoform to the plasma membrane of leaf stomatal guard cells (Remy *et al*., 2013).

Besides the plasma membrane and tonoplast, SUT proteins have been identified in an additional intracellular localization, namely the endomembrane system. AS a proof of concept, StSUT1, a plasma membrane transporter, has also been detected in endomembrane when expressed as GFP fusion protein. Its co-expression with ER marker protein has demonstrated its co-localization with the endoplasmic reticulum (Krügel *et al*., 2008). Moreover, StSUT1-GFP has labeled small motile vesicles with a size of about 200-500 nm (Liesche *et al*., 2010). Other sucrose transporters have been observed in endomembrane. StSUT4 from potato has been identified in both plasma

membrane and endomembrane in two distinct forms characterized by different molecular weight, corresponding to a full length protein (approximately 46 KDa), and to a truncated version, respectively (Chincinska *et al*., 2008). A similar result has been observed for NtSUT4 (Chincinska *et al*., 2013). Moreover, StSUT4-GFP was localized to the plasma membrane and to the membranes surrounding the nucleus, most likely the ER (Chincinska *et al*., 2008).

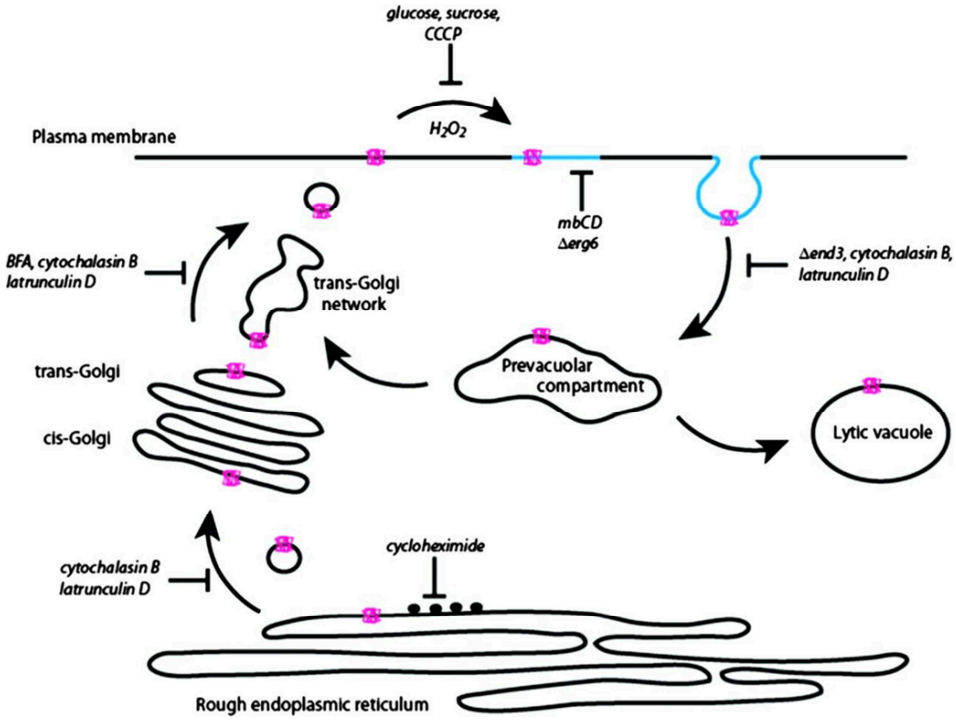


**Figure 55. Hypothetical model showing the posttranslational movement of SUT1 protein, involving ER, Golgi, vesicles, and Plasma Membrane (from Liesche *et al*., 2010)**

An hypothetical model has been suggested to describe the dynamic localization of sucrose transporter, in particular for those belonging to SUT1 subfamily, in different endomembranes (Figure 55). Sucrose transporters are targeted to the plasma membrane through the secretion pathway including synthesis of SUT proteins at the rough endoplasmic reticulum, transfer to the Golgi apparatus, and, after maturation, transport to the plasma membrane. The movement of SUT protein occurs in small vesicles. The sucrose transporters are located in the vesicles membrane and transferred to the plasma membrane through membranes fusion. The targeting of sucrose transporters is reversible. They can be internalized by endocytosis for degradation via lytic vacuoles, but also for constant recycling of the protein. The recycling might be important for transport activity regulation (Liesche *et al*., 2010).

**Redox regulation**

The activity of sucrose transporters is subjected to another type of protein regulation, depending on the redox conditions of the cell. This mechanism may likely be used by the plant for the rapid modification of carbohydrate transport in response to environmental changes (Slewinski and Braun, 2010).

It has been observed that in oxidative environment the targeting of sucrose transporter to the plasma membrane increases, more in detail the protein localizes in lipid-raft like structures. The membrane rafts domain have been defined as small (10-200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid enriched domains that compartmentalize cellular process (Pike, 2006). They are suggested to play a role in signaling, protein activity, endocytosis, oligomerization, degradation or transport of proteins (Grossmann *et al*., 2008). For example, the expression of SlSUT1-GFP in yeast, and the analysis of its localization under non reducing conditions revealed that the sucrose transporter distributes homogeneously on the plasma membrane and the intracellular membranes of yeast cell but, after oxidizing treatments, disappeared from the endomembrane and localized in defined regions of the plasma membrane, supporting the localization of SUT1 in lipid raft (Krügel *et al*., 2008).

Additionally, it has been well established that the redox environment influences the oligomeric state of the protein (Figure 56). For example, StSUT1 and SlSUT1 were shown to dimerize in a redox-dependent manner. Through native PAGE, as well as SDS-PAGE under non-reducing conditions and immunoprecipitation, it has been demonstrated that SlSUT1 and StSUT1 are able to form dimers in yeast, and plant (Krügel *et al*., 2008). It is possible that oxidative conditions increases the plasma membrane targeting in lipid-raft like domain, and therefore, the concentration in these domains may facilitate protein interactions. The SUT1 protein was detected as a dimer in the detergent-resistent membrane fraction isolated from potato plasma membrane supporting the localization of SUT1 in lipid raft (Krügel *et al*., 2008).

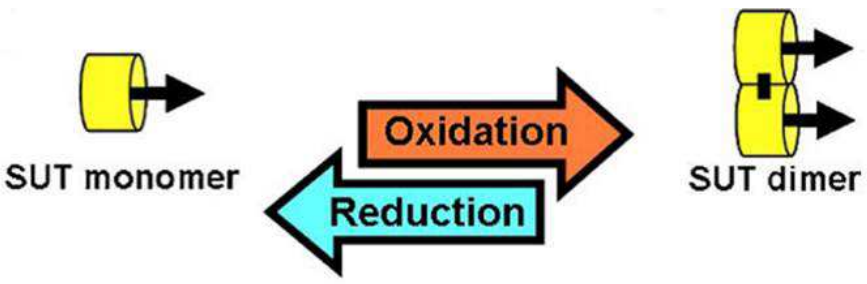


**Figure 56. Influence of the redox state in the formation of oligomers (modified from Slewinski and Braun, 2010).**

## 4.2. Methods

### 4.2.1. Isolation and cloning of *PpSUT* genes

Genes encoding sucrose transporters from peach were identify trough BLAST search on the available genome with SUT proteins from *Arabidopsis*. At least three isoforms have been recognized, i.e. ppa004033, ppa003041, and ppa004620 and renamed *PpSUT1*, *PpSUT2* and *PpSUT4*, respectively, according to the classification of SUT proteins adopted by Kühn and Grof (2010).

The complete DNA sequences of the three peach sucrose transporters (*PpSUT*) encoding genes were cloned into different vectors using the gateway technology (Invitrogen, Life Technologies), an easy system based on the site-specific recombination properties of bacteriophage lambda (Landy, 1989). Shortly, the gateway technology is constituted by two different reactions catalyzed by distinct clonase enzymes, the BP Clonase and LR Clonase that recombine attB/attP and attL/attR, respectively. The BP reaction allows the substitution of an attB-DNA fragment with a selective marker gene (ccdB, gene encoding an inhibitor of *E. coli* growth) flanked by attP sequence situated in a donor vector, producing an ENTRY clone carrying the gene of interest flanked by attL sites (Figure 57). With the LR reaction, the gene is transferred from ENTRY clone to a destination vector, i.e. an expression vector carrying the attR sites, through the recombination between attL and attR sites catalyze by LR Clonase enzyme (Figure 58).


**Figure 57. Reaction catalysed by BP Clonase enzyme. The recombination between attB and attP sites leads to move the gene of interest into donor vector.**


**Figure 58. Reaction catalysed by LR Clonase enzyme. Through the recombination between attL and attR, the LR Clonase is able to transfer the gene of interest from entry vector to destination vector.**

Each gene encoding a sucrose transporter from peach was cloned into the pDONR 207 gateway vector (Figure 59), either removing or retaining the stop codon, in order to produce native or C-terminal fusion protein.

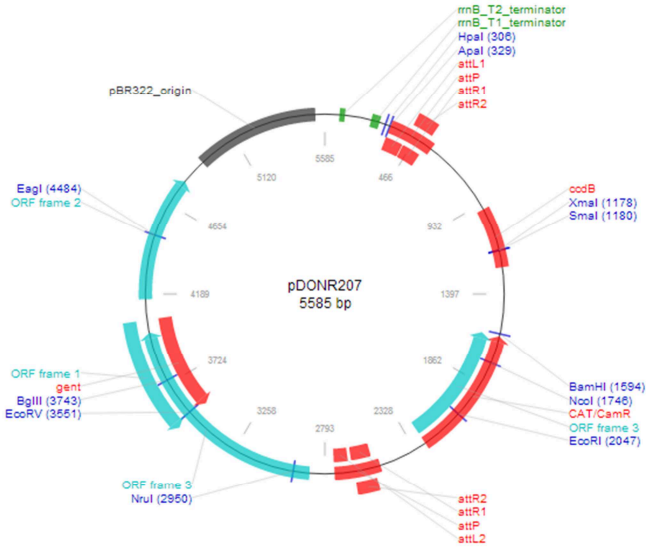


**Figure 59. Map of pDONR 207 vector.**

Firstly, to generate PCR products suitable for use as a substrate in the BP reaction, both attB sites (attB1 and attB2) were incorporated directly into the *PpSUT* DNA sequences by performing a double step PCR. Two different couples of primers were employed: the forward and reverse gene specific primers linked to 12bp of the attB1 and attB2 sequence, respectively, and the attB adapter primers to complete the attB sequences. All primers are listed in Table 5.

**Table 5. Primers used in the double step PCR for the incorporation of attB sites to the *PpSUT* sequences (attB1 at 5'-terminal and attB2 at 3'-terminal). Each construct was amplified with two different reverse primers in order to maintain or to remove the stop codon. In grey the sequences of attB adapter.**

| primer name | primer sequence |
|---|---|
| attB1-*PpSUT1* forward | AAAAAGCAGGCTTCATGGAAATTGAAACCGCG |
| attB2-*PpSUT1* reverse | AGAAAGCTGGGTAATGAAACCCAGCAGCTATAGG |
| attB2-*PpSUT1* reverse with stop | AGAAAGCTGGGTATCAATGAAACCCAGCAGCTATAG |
| attB1-*PpSUT2* forward | AAAAAGCAGGCTCCATGGCGGGGAAGACGGAC |
| attB2-*PpSUT2* reverse | AGAAAGCTGGGTAGCCAAAGTGAAAACCAGTTGAC |
| attB2-*PpSUT2* reverse with stop | AGAAAGCTGGGTATTAGCCAAAGTGAAAACCAGTTG |
| attB1-*PpSUT4* forward | AAAAAGCAGGCTGCATGCCAGCACCAGAAGCAG |
| attB2-*PpSUT4* reverse | AGAAAGCTGGGTATGTGAAAGCTCTGGGCTTC |
| attB2-*PpSUT4* reverse with stop | AGAAAGCTGGGTCTCATGTGAAAGCTCTGGGC |
| attB1 adapter | GGGGACAAGTTTGTACAAAAAAGCAGGCT |
| attB2 adapter | GGGGACCACTTTGTACAAGAAAGCTGGGT |

In the first step the PCR reactions were composed of 4 µl cDNA (10ng/µl), 0.2 µl Phusion® High-Fidelity DNA Polymerase (Finnzymes, NewEngland BioLabs), 4 µl 5X Phusion HF Reaction Buffer, 0.4 µl dNTPs (10 µM, 2mM each), 0.4 µl attB1-gene specific primer forward (10 µM), 0.4 µl attB2-gene specific primer reverse (10 µM), 0.6 µl DMSO and 10 µl $H_2O$. Each sample was incubated in a

thermal cycler (T3 Thermocycler, Biometra) according to the following scheme: one step at 98°C for 30 sec, 10 cycles at: 98°C for 10 sec, primers specific annealing temperature for 30 sec, 72°C for 30 sec/Kb, and finally one step at 72°C for 5 min. The products were directly used as a template in the second step of PCR in order to complete the attB sequence. The reactions were constituted of 10 µl of first PCR, 10 µl 5X Phusion HF Reaction Buffer, 1 µl dNTPs (10 µM, 2mM each), 1 µl attB1 adapter primer (40 µM), 1 µl attB2 adapter primer (40 µM), 0.5 µl Phusion® High-Fidelity DNA Polymerase (Finnzymes, NewEngland BioLabs), 1.5 µl DMSO and 25.5 µl $H_2O$. The PCR thermocycler was programmed as follows: 1 cycle at 98°C for 30 sec, 5 cycles at: 98°C for 10 sec, 45°C for 30 sec, 72°C for 30 sec/Kb, 20 cycles at: 98°C for 10 sec, 55°C for 30 sec, 72°C for 30 sec/Kb and a final step at 72°C for 5 min. The annealing temperatures and the extension times specific for each construct are listed in Table 6.

**Table 6. Specific annealing temperatures and extensions times employed in the double step PCR.**

| gene name | $T_{annealing}$ | extension |
|---|---|---|
| *PpSUT1* without stop | 58°C | 50 sec |
| *PpSUT2* without stop | 64°C | 60 sec |
| *PpSUT4* without stop | 58°C | 60 sec |
| *PpSUT1* with stop | 58°C | 50 sec |
| *PpSUT2* with stop | 62°C | 60 sec |
| *PpSUT4* with stop | 62°C | 60 sec |

Then, the attB-PCR products were purified from 1% agarose gel by Invisorb® Spin DNA extraction Kit (Invitek) according to the manufacturer's instruction and quantified by Nanodrop ND2000 (Thermo Scientific). Subsequently, the purified attB-DNA fragments were cloned into pDONR 207 vector by BP recombination reaction. The following components were mixed: 300 ng attB-PCR product, 300 ng pDONR 207 vector, 4 µl 5X BP Clonase™ reaction buffer, TE buffer pH 8 to 16 µl and 4 µl BP Clonase™ enzyme mix. Samples were incubated 2 h at 25°C. Hence, reactions were stopped adding 2 µl Proteinase K and incubating at 37°C for 10 min.

Subsequently, chemically competent *E. coli* cells (strain DH5α) were transformed pipetting 10 µl of each reaction directly into an aliquot of cells. Samples were incubated 30 min on ice, heat shocked at 42°C for 90 sec and then immediately transferred on ice for 3 min. Before incubating at 37°C for 1 h with 250 rpm shaking, 500 µl LB medium (10 g Bacto-tryptone, 5 g yeast extract and 5 g NaCl) without antibiotics were added. Finally, transformed cells were spread on LB agar plates containing 15 µg/ml gentamicin. Plates were incubated overnight at 37°C. Then, for each construct

six colonies were chosen and grown on 5 ml LB medium with 15 µg/ml gentamicin at 37°C overnight with 250 rpm shaking.

Plasmids were extracted from transformed *E. coli*. Cells were harvested by centrifugation at 12.000 rpm for 2 min from 4 ml of bacterial culture. LB medium was discarded and bacterial pellet resuspended in 300 µl Buffer P1 (2mM Tris-HCl, 0.4 mM EDTA·2$H_2O$, 100µg/ml RNAse pH 8.0). Then, 300 µl Buffer P2 (8 mM NaOH, 1% SDS) were added in order to lyse cells and tubes gently inverted six times. Samples were incubated at room temperature for 5 min and, after the addition of 300 µl Buffer P3 (0.12 M potassium acetate, 11% acetic acid, pH 5.5), maintained 10 min on ice. The cellular debris were collected by centrifugation at 14.000 rpm for 20 min at 4°C. Supernatant containing nucleic acids was recovered. The plasmids were precipitated mixing 0.7 volume of isopropanol and incubating samples 2 min at room temperature. Plasmids were collected by centrifugation at 14.000 rpm for 20 min at 4°C. Supernatant was removed and pellets washed with 500 µl ethanol 75%. After spinning at 14.000 rpm for 5 min at 4°C, ethanol was discarded and pellets dried at 42°C, then resuspended in 30-50 µl $H_2O$ incubating tubes at 42°C for 5 min. Plasmid concentration was estimated by Nanodrop ND2000 (Thermo Scientific) and by comparison with standard DNA on 1% agarose gel. Cloning was checked through PCR using DreamTaq Green DNA Polymerase (Thermo Scientific). A mix of 0.5 µl entry clone (pDONR 207 vector carrying PpSUT gene), 2 µl 10X DreamTaq buffer, 0.4 µl dNTPs (10 µM, 2mM each), 0.4 µl gene specific primer forward 10 µM, 0.4 µl gene specific primer reverse, 0.125 µl DreamTaq Green DNA Polymerase and 16.175 µl $H_2O$ was prepared and incubated in a thermocycler (T3 Thermocycler, Biometra) according to the following scheme: 1 step at 95°C for 3 min, 30 cycles at 95°C for 30 sec, primers specific $T_m$ for 30 sec and 72°C for 2 min, and finally 1 step at 72°C for 5 min. The specific $T_m$ adopted are listed in Table 6. PCR products were checked by electrophoresis on 1% agarose gel. Then, one plasmid per construct was purified through MSB® Spin PCRapace (Invitek) according to the manufacturer's instructions and an aliquot was used to check products by sequencing with primers specific for pDONR 207 vector flanking the insertion site (pDONR207 forward: TCGCGTTAACGCTAGCATGGATCTC; pDONR207 reverse: GTAACATCAGATTTTGAGACAC).

After cloning into donor vector, *PpSUT* genes were transferred in different destination vector, according to the type of protein expressed and the system of expression, by a LR recombination reaction catalysed by LR Clonase. Each LR reaction was composed of 150 ng entry clone, 150 ng destination vector, TE buffer pH 8 to 8 µl and 2 µl LR Clonase™ II enzyme. Samples were incubated 2 h 30 min at 25°C. Then, the activity of LR Clonase was stopped by addition of 2 µl of Proteinase K

and by incubation at 37°C for 10 min. Each LR reaction was used to transform chemically competent *E. coli* cells (strain DH5α) as described above. Six colonies were chosen and grown on 5 ml LB with appropriate antibiotics at 37°C overnight at 250 rpm. The expression vectors (destination vector containing *PpSUT* gene) were extracted as previously described and quantified by Nanodrop ND2000 (Thermo Scientific). The constructs were checked by PCR using DreamTaq Green DNA Polymerase (Thermo Scientific) (see above). Finally, plasmids were cleaned through MSB® Spin PCRapace (Invitek) according to the manufacturer's instructions.

In Table 7 the distinct types of destination vectors adopted in the different experiments are summarized.

**Table 7. Distinct destination vector used in relation to the type of protein expressed in the different experiments performed.**

| | assay type | type of expressed protein | gene sequence | antibiotic resistance |
|---|---|---|---|---|
| **PK7YWG2 and PB7CWG2** | subcellular localization | C-terminal fusion protein | PpSUT gene without stop codon | Spectinomycin |
| **pDR196** | yeast functional analysis | native protein | PpSUT gene with stop codon | Ampicillin |
| **metXCgate and pNXgate** | split-ubiquitin assay | C-terminal fusion protein | PpSUT gene without stop codon | Kanamycin |

### 4.2.2. Functional analysis in yeast

The function of sucrose transporters from peach was tested by two different experiments: the yeast complementation assay and the esculin uptake assay.

The complete CDS of *PpSUT* genes cloned into pDR196 has been used to transform two different mutant strains of *Saccharomyces cerevisiae*, named 18GAS and EBY.SL (Wieczorke *et al*., 1999). As Susy7 strain (Riesmeier *et al*., 1992), both are unable to grow on media containing solely sucrose as energy source, but they have a reduced background as compared to Susy7, because of the deletion of 18 hexose transporters. Moreover, EBY.SL lacks of two other genes encoding maltose transporters.

**Yeast transformation**

For each yeast strain, a single colony was grown on 25 ml of SD liquid medium (1.7 g $L^{-1}$ Yeast Nitrogen Base [YNB] without amino acids and without ammonium sulfate [MPbio], 5 g $L^{-1}$ Ammonium sulfate, 20 g $L^{-1}$ maltose, 0.79 g $L^{-1}$ Complete Supplement Mixture [CSM, MPbio]) overnight at 30°C, with shacking (300 rpm), to an $OD_{600}$ of 0.8. Cells were collected by centrifugation at 2000 rpm for 5 min and washed in 20 ml of 1X TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). After a second centrifugation at 2000 rpm for 5 min, pellet was resuspended in 0.5-1 ml of Mix1 (0.1 mM Lithium Acetate, 0.5X TE buffer, 1 M Sorbitol) and incubated at room temperature for 20 min. Then four solutions containing different vectors were prepared: 5 µl vector (empty pDR196 vector, pDR196 carrying PpSUT1, PpSUT2 and PpSUT4), 5 µl denatured Salmon Sperm, 40 µl yeast cells resuspended in Mix1 and 230 µl Mix2 (0.1 M Lithium Acetate, 1X TE buffer, 40% PEG 3350). After mixing by inverting tubes several times, cells were incubated at 30°C for 30 min without shacking; 30 µl DMSO were added and the solutions were incubated at 42°C for 7 min. Finally, cells were plated on solid selective medium (SD medium without URA containing 1.7% Yeast agar) and grown for 2-3 days at 30°C.

**Yeast complementation assay**

After transformation one colony for each EBY.SL and 18GAS transformed strain was grown on 2 ml of liquid SD-URA containing maltose in a 30°C shaker (300 rpm) overnight (approximately 16 hours). Cells were collected by centrifugation at 2000 rpm for 5 min, washed with sterile water and resuspended in 2 ml of SD-URA medium. The $OD_{600}$ was checked and adjusted to 1, then for each sample three dilutions were prepared (0.1, 0.01 and 0.001). Finally, 5µl of each dilution were placed on two different selective medium: SD-URA containing 2% sucrose or 2% maltose as a unique carbon source. Plates were incubated at 30°C until yeast growth. Images were taken by scanning of the plates.

**Determination of yeast transformants growth curve**

In order to compare growth rate of yeast expressing PpSUT proteins, transformants of 18GAS mutant strain expressing sucrose transporters from peach, were grown on two different SD-URA medium, containing maltose or sucrose as a unique carbon source and the $OD_{600}$ was monitored over time. Transformants were firstly grown on 2 ml SD-URA medium containing maltose at 30°C overnight at 300 rpm. Then, the $OD_{600}$ was checked and each strain diluted to an $OD_{600}$ of 0.1.

Subsequently, cells were collected by centrifugation at 2000 rpm for 5 min and resuspended in two different SD-URA medium, supplemented with maltose or sucrose. Each sample was grown in triplicate at 30°C into 96-well transparent plate and the $OD_{600}$ was measured every 30 min for 48 hours by means of plate reader (SpectraMax M2, Molecular Devices) in order to plot a growth curve.

**Esculin uptake assay**

The same yeast transformants analyzed by yeast complementation assay were checked by esculin uptake experiment, a novel assay developed by Gora *et al*. (2012) to evaluate the functionality of sucrose transporters. The test is based on the ability of type 1 SUT transporters to transport different kind of molecules including the highly fluorescent compound esculin (6,7-dihydroxycoumarin β-D-glucoside). A yeast cell expressing a functional sucrose transporter is able to take up and accumulate esculin becoming highly fluorescent. The signal can be easily detected by fluorescence microscopy.

Tubes containing 2 ml of SD-URA medium supplemented with maltose were inoculated with yeast transformants and incubated overnight at 30°C with shacking (300 rpm). Cells were collected by centrifugation at 1300 g for 5 min. Then supernatant was discarded and replaced with 1 ml of phosphate buffer (25 mM $Na_2HPO_4$, pH 4) containing 1 mM of fluorescent esculin substrate. Cells were resuspended by vortex and incubated at 30°C with shacking (300 rpm) for 1 hour. To remove esculin, tubes were centrifuged at 1300 g for 5 min, after which the buffer was discarded. Cells were washed with 2 ml of phosphate buffer without esculin and resuspended in 1.5 ml of the same buffer by gently pipetting. 10µl of each sample were placed on a slide in order to observe the cells by confocal microscope (TCS SP2, Leica). Esculin was excited at 367 nm and signal detected in the visible region, at 454 nm.

**Esculin quantification**

In order to evaluate the pH-dependence for PpSUT transport activity, the uptake of esculin was measured in three different conditions of pH (4, 5.5 and 7). Mutant EBY.SL and 18GAS strains expressing PpSUT proteins were treated with esculin as previously described (see 1.2.4). The amount of esculin absorbed by yeast transformants was quantified measuring the fluorescence emission at 454 nm by plate reader (SpectraMax M2, Molecular Devices). Samples were prepared

in a 96-well black microtiter plate in triplicate. Cell density was determined by absorbance at 600 nm in order to calculate the relative fluorescence per unit of $OD_{600}$.

### 4.2.3. Split-Ubiquitin Membrane Yeast Two-Hybrid System

Because the ability of sucrose transporters to interact with each other has been observed in diverse plant species, a split-ubiquitin membrane yeast two-hybrid system (MYTH; Kittanakom *et al*., 2009) was performed with PpSUT proteins from *Prunus persica* in order to evaluate the putative capacity of sucrose transporters to form dimers in a different plant system. The conventional yeast two hybrid system (Fields and Song, 1989; Uetz and Hughes, 2000) has been widely used to detect protein-protein interactions, but because the detection system is based on the reconstruction of a transcription factor associated to the proteins analyzed and on its movement into the nucleus for the activation of a reporter gene, it cannot be applied for the study of plasma membrane dimers. For these reasons, the MYTH has been developed. The system involves the use of ubiquitin, a conserved 76 amino acids peptide implicated in the protein degradations via 26S proteasome. This protein has been split in two halves, the amino-terminal (Nub, aa 1-34) and the carboxy-terminal (Cub, aa 35-76). When expressed in the same cell, the two moieties can reconstitute the ubiquitin. Before using in the MYTH, they have been slightly modified. An artificial transcription factor has been fused to the Cub, while a substitution of the isoleucine in position 13 with a glycine in the N-terminal moiety (NubG) has been performed to reduce the affinity between the two halves preventing the spontaneous reassembly of ubiquitin. Then, the prey-protein is linked to N-terminal and the bait-protein to C-terminal. If these two peptides interact, Nub ad Cub moieties can reconstitute the pseudo-ubiquitin that can be recognized by specific ubiquitin protease. The proteases cleave after C-terminal of ubiquitin releasing the transcription factor which enters the nucleus and activates the reporter gene.

Genes encoding sucrose transporters from peach were cloned into two different vectors, metXCgate and pNXgate, in order to express PpSUT proteins in two different forms: as a C-terminal fusion protein to a C-terminal subdomain of ubiquitin (Cub) and as C-terminal fusion protein to a N-terminal moiety of ubiquitin (Nub). Every PpSUT-Cub protein was expressed together with each PpSUT-Nub protein in order to analysed all the possible combinations. Each PpSUT-Cub construct was transformed into L40 yeast strain (MATa ade3 trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-LacZ) as described in 1.2.1. The selection of transformed colonies was performed in a SD medium without leucine. A second transformation was made with the PpSUT-Cub

transformed L40 strains in order to introduce the PpSUT-Nub constructs. Yeast transformants were selected in a SD medium without leucine and tryptophan. Then, one colony for each double transformant was grown on liquid SD -Leu -Trp at 30°C overnight at 300 rpm. The $OD_{600}$ was measured and a drop of each sample (5 µl) of three different dilutions (OD= 0.1, 0.01, 0.001) was placed in a solid SE-medium without leucine, tryptophan, histidine and uracil supplemented with 2% glucose or 2% sucrose, with or without 3AT in order to evaluated the putative protein interaction.

### 4.2.4. Subcellular localization of C-terminal tagged PpSUT proteins

To localize sucrose transporters from peach at subcellular level, each *PpSUT* DNA sequence without codon stop was cloned into two different gateway expression vectors, PK7YWG2 and PB7CWG2 (Karimi *et al*., 2002) in order to overexpress C-terminal fusion peptides tagged with two fluorescent proteins, the Yellow Fluorescent Protein (YFP) and Cyan Fluorescent Protein (CFP) in *Nicotiana benthamiana* leaves through a transient transformation mediated by *Agrobacterium*. The purpose of the experiment was to co-infiltrate leaves with two different constructs, one carrying *PpSUT* genes from peach and the other holding a plasma membrane or a vacuolar marker. The two proteins expressed in the same cells were labelled with different fluorescent tags and the two signals, observed by means of confocal microscope, were compared in order to assess the co-localization of PpSUT protein with one protein marker.

First of all, *Agrobacterium* cells (strain GV2260) were transformed with *PpSUT* constructs by electroporation. For each construct, 1 µl of cleaned plasmid was mixed with 20 µl of electrochemically competent *Agrobacterium* cells and transferred into electroporation cuvettes (VWR). The cuvettes were placed on MicroPulser™ Electroporator (BIO RAD) and an electrical impulse was passed to allow the insertion of plasmid. Then, 500 µl of YEB medium (5 g casein enzymatic hydrolysate from bovine, 1 g yeast, 5 g sucrose, 0.492 g $MgSO_4 \cdot 7H_2O$ in 1 L of milliQ water, pH 7.2) without antibiotics were added, the solution was transferred in new 2 ml tubes and incubated at 30°C for 2 h 30 min at 250 rpm. Finally, 50 µl and all cells, collected by centrifugation at 3500 rpm for 2 min, were spread on agar-YEB plates containing 50 µg/ml spectinomycin (resistance carried by gateway vectors), 100 µg/ml rifampicin and 100 µg/ml ampicillin (both resistance located in *Agrobacterium* genome) as a selective agents. Cells were grown at 30°C for two days.

Subsequently, transformed *Agrobacterium* was cultivated in liquid medium in order to collect and prepare cells for transient transformation of *Nicotiana benthamiana* leaves. Together with the strains carrying PpSUT proteins, other three transformants holding constructs for the expression of CBL1, PTR2 and P19 were grown. CBL1, that is fused to OFP (orange fluorescent protein) is a calcineurin B-Like Protein that has been localized to plasma membrane (Batistič *et al*., 2008), PTR2 tagged with YFP is a peptide transporter that has been described as a tonoplast located protein (Komarova *et al*., 2012), whereas P19 is a viral-encoded suppressor of post-transcriptional gene silencing, a general plant response that limits the efficiency of Agrobacterium-mediated transient expression. The function of P19 is to enhance protein transient expression (Voinnet *et al*., 2003). Firstly, a pre-culture was prepared inoculating with one colony for each transformant 2 ml of YEB medium containing appropriate antibiotics and incubating cells at 30°C for 1 day at 250 rpm. Pre-cultures were used to inoculate 50 ml of YEB medium containing the appropriate antibiotics and 10 µl acetosyringone (100mM in DMSO; 3',5'-Dimethoxy-4'-hydroxyacetophenone) prepared fresh. *Agrobacterium* cells were grown once again at 30°C overnight at 250 rpm. Hence, cells were collected by centrifugation at 3500 rpm for 15 min, medium was discarded and pellets washed with deionized sterile $H_2O$. After a second centrifugation at 3500 rpm for 15 min, $H_2O$ was removed and pellets resuspended in 10 ml of infiltration medium (10 mM MgCl, 10 mM MES, 100 µM acetosyringone, pH 5.7). Then, cells were incubated for 2 h at room temperature. An aliquot of each transformant was used to measure the optic density at λ=600 by spectrophotometer. Finally, the solutions for infiltration were prepared mixing constructs carrying *PpSUT* with one protein marker and P19, all diluted at an $OD_{600}$=0.5. The infiltration was performed by means of a syringe without needle, inoculating the underside of *Nicotiana benthamiana* leaves. After three days from infiltration, small discs (about 0.5 mm) of transformed leaves were collected and observed at confocal microscope (TCS SP2, Leica).

### 4.2.5. SDS-PAGE and Westen-blot

In order to check the expression of PpSUT proteins in transient transformed *Nicotiana benthamiana* leaves, a western-blot analysis was performed using a commercial antibody against GFP that is also able to recognized YFP or CFP.

Proteins were extracted from 200 mg of transformed leaves in two different conditions, in the presence or absence of 5 mM DTT, a strong reducing agent. After tissue grinding in a fine powder, 600 µl of protein extraction buffer (30% sucrose, 25 mM EDTA, protease inhibitors [Roche], 250

mM Tris-HCl pH 8.5) with or without DTT were added. Cell debris were collected by centrifugation at 4°C for 10 min at 5000 g and supernatant transferred in new tubes. Proteins were precipitated by a second centrifugation at 4°C for 20 min at full speed. Supernatant was discarded and pellet resuspended in 30-40 µl of 4X Laemmli buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 20% Gycerin) supplemented or not with 20 mM DTT. Samples were incubated for 20 min at 37°C, then centrifuged for 2 min at 7000 rpm. Finally, 20 µl of each sample were loaded in a 12.5 % polyacrylamide gel. Run was made for 1h at 130 V.

Subsequently, protein gel blotting was performed by a semidry electroblotter (Bio Rad) in transfer buffer (12.5 mM Tris, 100 mM Glycin, 0.034 mM SDS) supplemented with methanol and incubated for 1 h at 40 mA. Membrane was washed with 1X TBS (12.4 mM Tris, 1.5 M NaCl, pH 7.5) and incubated in blocking buffer (3% milch powder in 1X TBST [1X TBS + 0.02 % Tween]) overnight at 4°C in slow agitation. Then, it was washed once in 1X TBST for 10 min, twice in 1X TBS for 5 min and subsequently incubated for 2 h in 1X TBS with 1% milchpulver containing a commercial antibody anti-GFP produced in rabbit (Sigma) diluted 1:1000. Membrane was washed once in 1X TBST and three times in 1X TBS for 10 min each, then incubated for 1h in 1X TBS + 1% milchpulver added with anti-rabbit secondary antibodies (Sigma) conjugate to an alkaline phosphatase diluted 1:5000, after which one wash in 1X TBST and two washing in 1X TBS for 10 min each were performed. Finally, the substrate (luminol) for the reaction of chemiluminescence was added and development of luminescence detected by imaging system (Stella, Raytest).

## 4.3. Results

### 4.3.1. Functional analysis of PpSUT proteins in heterologous system

Three genes encoding sucrose transporters were identified in peach genome through a blast search with SUT proteins from Arabidopsis. In order to study the functionality of these genes, the complete CDS were cloned into pDR196 vector and expressed in two different mutant *S. cerevisiae* strains (EBY.SL and 18GAS). The transformed strains were subsequently analyzed by two distinct types of experiments: the yeast complementation assay and the esculin uptake assay.

**Yeast complementation assay**

In the yeast complementation assay, the sucrose transport capacity of PpSUT proteins has been tested through the heterologous expression in *S. cerevisiae* mutant strains, called EBY.SL and 18GAS, that are unable to grow on medium containing sucrose as sole carbon source (Wieczorke *et al*., 1999), because lacking in a system for directly taking up sucrose and for hydrolyzing it outside the cell. The function of PpSUT proteins as a sucrose transporter was confirmed by growing the mutant yeast strains, transformed with a vector carrying *PpSUT* gene, on a selective medium supplemented with sucrose as carbon source. This indicated that yeast takes up the disaccharide, and uses it as energy source to sustain metabolism and growth of cells.

After 10 days of incubation at 30°C, the expression of PpSUT1 and PpSUT4 allowed 18GAS yeast strain to grow on selective media added with sucrose (Figure 60A); this result provides an indication that both proteins are able to transport the disaccharide inside yeast cells, and they are functional sucrose transporters. Moreover, yeast transformed with construct carrying *PpSUT1* gene showed better growth compared to that transformed with vector carrying *PpSUT4* gene. In fact, the colonies produced by yeast expressing PpSUT1 were bigger and more compact, whereas those produced by yeast expressing PpSUT4 were smaller and less dense. On the contrary, the expression of PpSUT2 protein did not resume the phenotype of mutant strain, neither at high concentration (OD=0.1). The growth of yeast transformed with pDR196 vector without insert, as a negative control, confirmed the assay reliability. Furthermore, all the strains were grown on media containing maltose as a carbohydrate source, confirming the viability of transformants (Figure 60B).

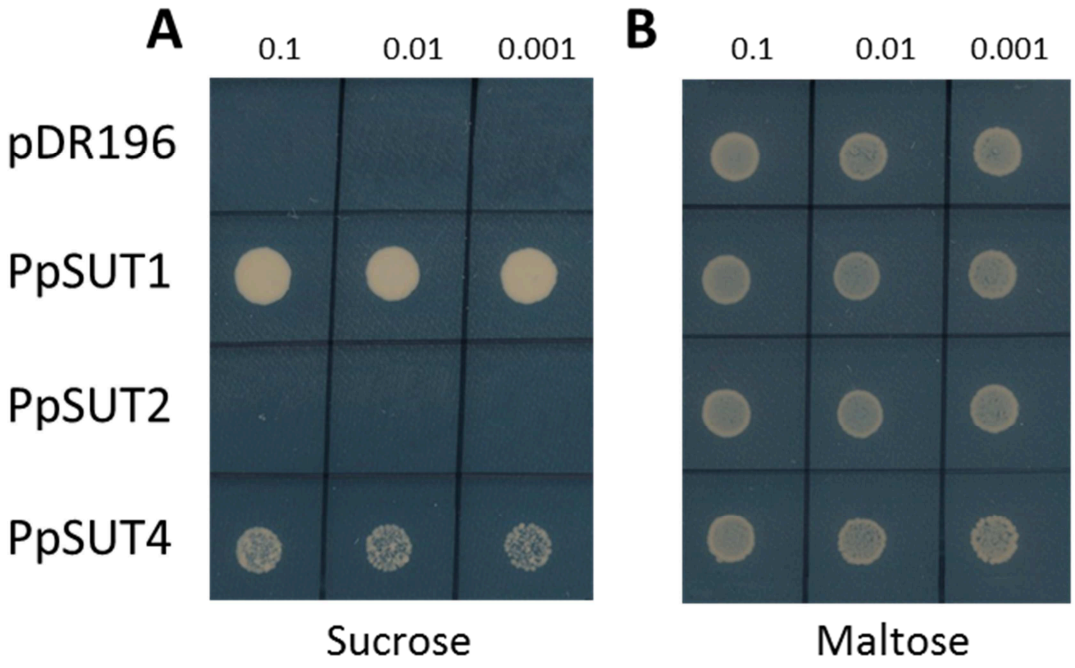


**Figure 60. Growth of yeast strains 18 GAS transformed with PpSUT1, PpSUT2, PpSUT4 and empty vector (pDR196), on 2% sucrose (A) and 2% maltose (B) as exclusive carbon source, after 10 days of incubation at 30°C. Each strain was plated in three different concentrations (OD: 0.1, 0.01 and 0.001).**

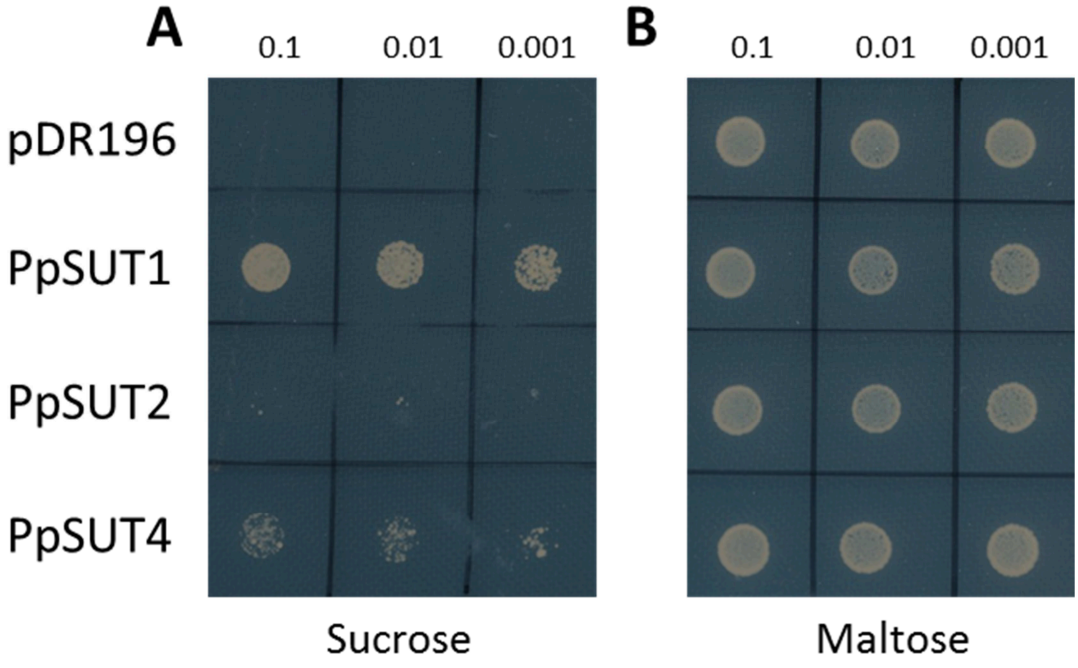


**Figure 61. Growth of yeast strains EBY.SL transformed with PpSUT1, PpSUT2, PpSUT4 and empty vector (pDR196), on 2% sucrose (A) and 2% maltose (B) as the exclusive carbon source, after 10 days of incubation at 30°C. Each strain was plated in three different concentrations (OD: 0.1, 0.01 and 0.001).**

The yeast complementation assay, performed with sucrose-uptake deficient EBY.SL strain expressing PpSUT, confirmed the results obtained with 18GAS strain (Figure 61A). In fact, also in

this case, the presence of PpSUT1 allowed yeast cells to grow faster than yeast expressing PpSUT4. The presence of PpSUT2 did not allowed the growth of yeast colonies. The negative control, EBY.SL transformed with empty pDR196 vector, confirmed the assay reliability, whereas the growth of transformants in maltose plate confirmed the viability of cells (Figure 61B).

**Growth curve of transformed yeasts**

In order to evaluate whether the expression of PpSUT belonging to distinct subfamily could determine a different growth rate of mutant yeast cells, transformants of 18GAS strain, that grow faster than EBY.SL, were grown at 30°C on two different liquid medium, one supplemented with maltose and the other with sucrose, and the increased of $OD_{600}$ over time. The growth curve of yeast strains incubated in the culture medium supplemented with maltose showed the typical yeast growth pattern described by a simple sigmoid, characterized by three phases (lag-phase, exponential-phase and plateau-phase). All strains divided quite rapidly, reaching the plateau phase at about 25 h (Figure 62).

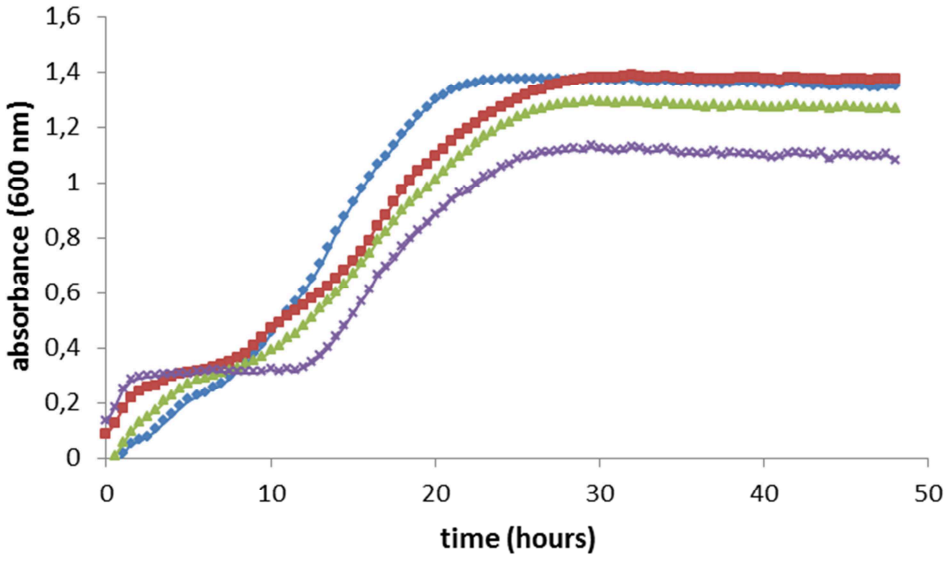


**Figure 62. Growth curve, expressed as absorbance variation, of yeast strain 18 GAS transformed with pDR196 (●), PpSUT1 (●), PpSUT2 (●) and PpSUT4 (●) inoculated in a medium supplemented with maltose.**

On the contrary, yeast transformants in the presence of sucrose (Figure 63), showed a slow growth. In fact, after an incubation of 48 hours, the $OD_{600}$ remained at low levels, and only yeast strain expressing PpSUT1 was able to enter in the exponential phase. The difference could be attributed to higher sucrose affinity for the substrate.

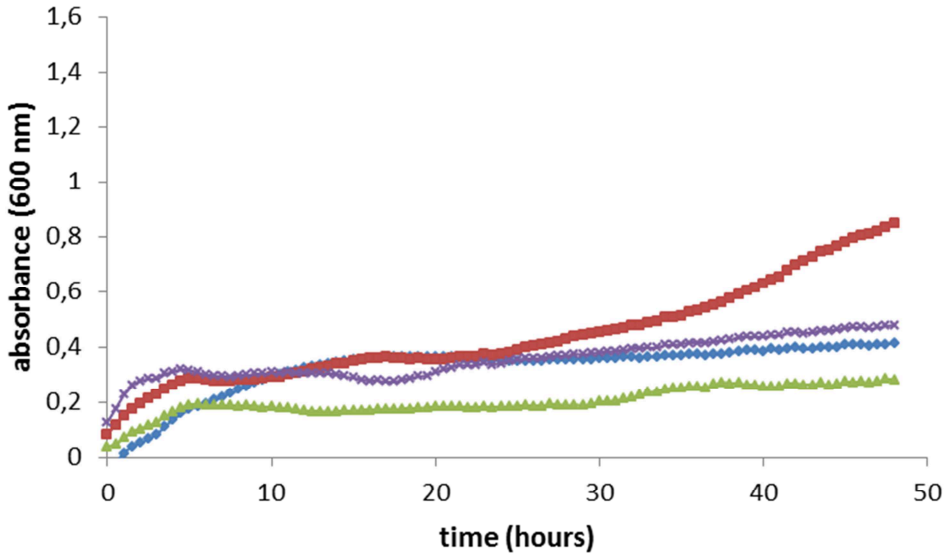


**Figure 63. Growth curve of yeast strain 18 GAS transformed with pDR196 (●), PpSUT1 (●), PpSUT2 (●) and PpSUT4 (●) inoculated in a medium supplemented with sucrose.**

### Esculin uptake assay

The functionality of sucrose transporters from *Prunus persica*, yeast strain EBY.SL and 18GAS expressing PpSUT sucrose transporters was further analyzed by means of the "esculin uptake assay". This novel approach is based on the ability of sucrose transporters, in particular those belonging to clade 1, to transport several kind of molecules, including the highly fluorescent molecule esculin (6,7-dihydroxycoumarin β-D-glucoside) (Gora *et al.*, 2012). Yeast cells expressing a functional sucrose transporter are able to accumulate esculin and become fluorescent.

The observation of yeast transformants of mutant strain 18GAS by confocal microscope, after incubation in a solution supplemented with esculin, revealed that the presence of PpSUT1 within yeast cells allowed the uptake of the fluorescent dye (Figure 64). In fact, cells became fluorescent confirming the functionality of the protein. However, the overlapping (Figure 64C) of pictures taken under fluorescent (Figure 64A), and transmission light (Figure 64B) pointed out that not all cells absorbed the fluorescent dye. This may be due to a down-regulation of the promoter controlling the expression of gene cloned in the pDR196 vector, when cells begin to enter stationary phase (Gasch *et al.*, 2000; Gora *et al.*, 2012). On the contrary, no fluorescence was detected in yeast cells expressing both PpSUT2 and PpSUT4 transporters. The negative control confirms the incapacity of 18GAS to import esculin in the absence of sucrose transporters expression.

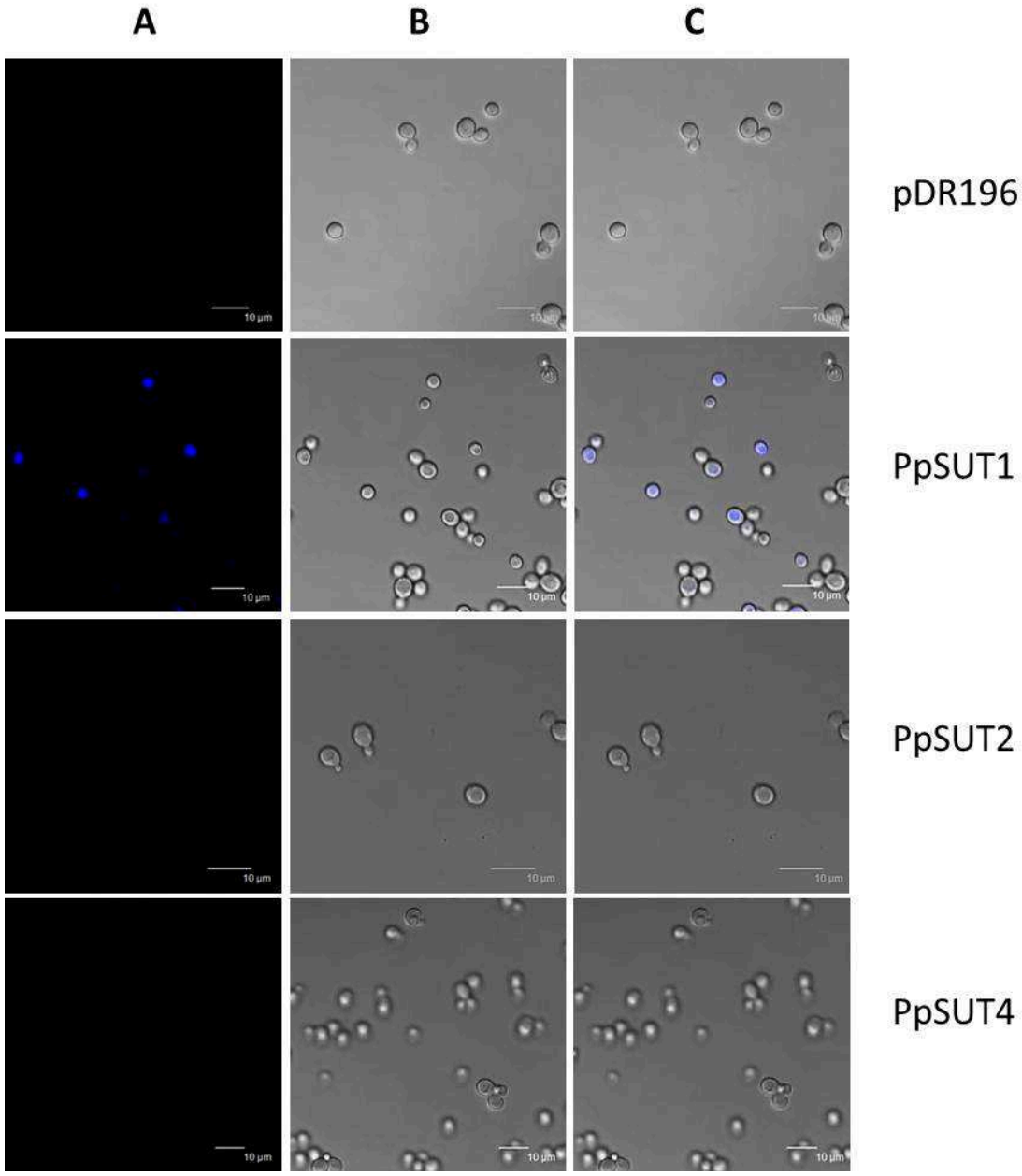


**Figure 64. Esculin uptake by yeast 18GAS transformed with PpSUT1, PpSUT2, PpSUT4 and empty vector (pDR196). Pictures taken by confocal microscopy of esculin emission (column A), transmission light (column B) and overlapping of both esculin emission and transmission pictures (column C). The images represent a maximum projection of z-stacks.**

The esculin uptake assay confirmed the characteristic of sucrose transporters belonging to SUT1 clade to be less selective for sucrose, being able to transport other glucosides, in this case the coumarin β-glucoside esculin (Sivitz *et al*., 2007).

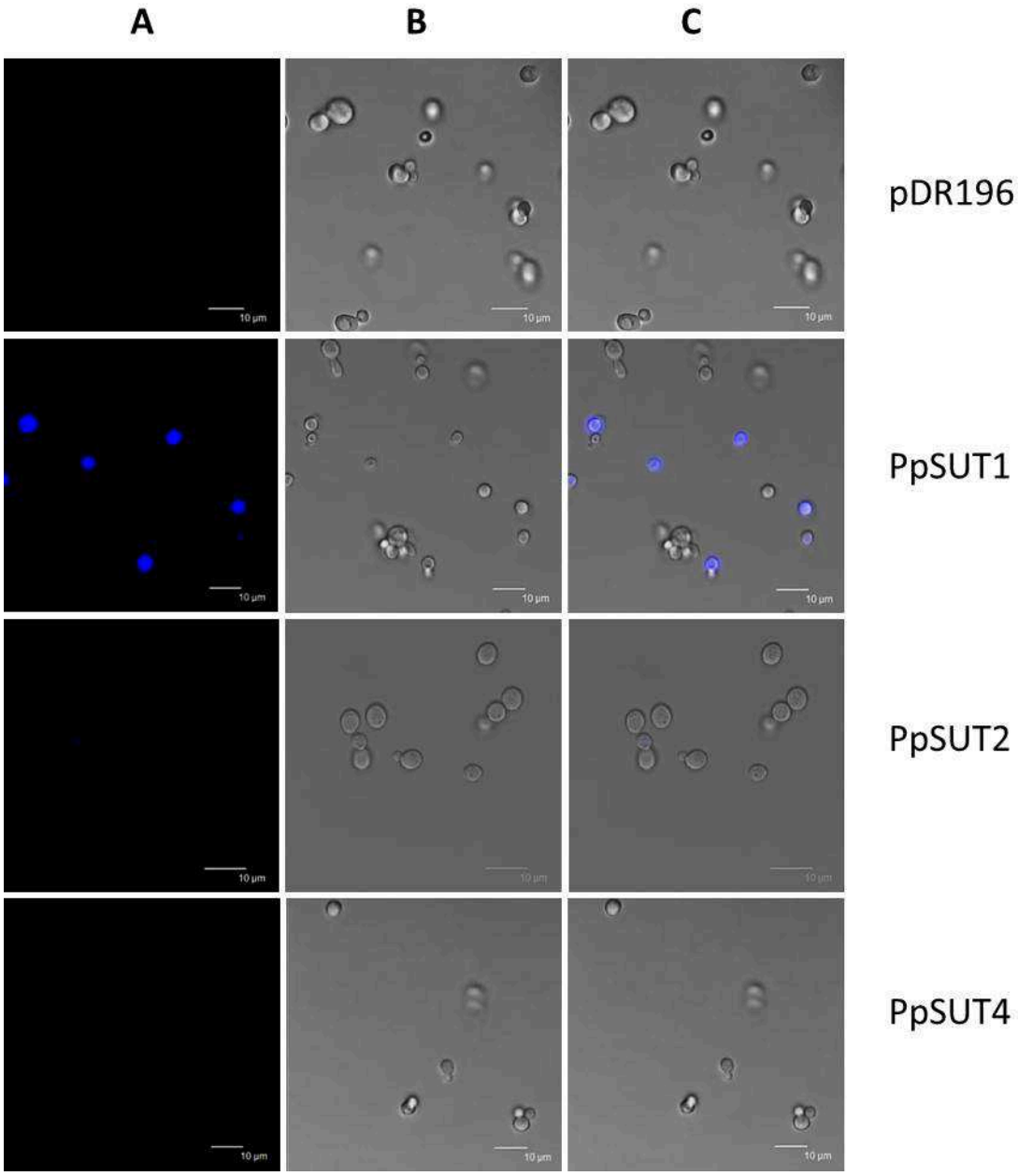


**Figure 65. Esculin uptake by yeast EBY.SL transformed with PpSUT1, PpSUT2, PpSUT4 and empty vector (pDR196). Pictures taken by confocal microscopy of esculin emission (column A), transmission light (column B) and overlapping of both esculin emission and transmission pictures (column C). The images represent a maximum projection of z-stacks.**

The same result was obtained analyzing PpSUT proteins expressed in EBY.SL mutant strain (Figure 65). In fact, the blue fluorescence was detected in the transformant expressing PpSUT1, whereas the presence of both PpSUT2 and PpSUT4 proteins did not allow the transport of esculin into yeast cells. Yeast strain that did not express any SUT proteins confirmed the inability of yeast EBY.S to transport esculin.

**Esculin quantification**

So far, all sucrose transporters have been characterized as $H^+$/symporters, therefore their activity is strictly connected to the proton gradient across the membranes. In order to evaluate the pH-dependence of PpSUT proteins transport activity, the uptake of esculin was quantified in three different pH conditions, i.e. 4, 5.5 and 7. The results of quantification are shown in Figure 66 and Figure 67, representing the esculin uptake in 18GAS and EBY.SL transformed strains, respectively. In both cases, yeast cells expressing PpSUT1 accumulated high amount of esculin at pH 4, while the quantity of fluorescent dye taken up at pH 5.5 was lower, and almost undetectable at pH 7. This is consistent with the pH-dependence of sucrose transporters activity. On the contrary, the amount of esculin absorbed by yeast cells expressing PpSUT2 and PpSUT4 was undetectable, and it was not possible to establish the influence of different pH conditions on the activity of these transporters. As expected, yeast strain transformed with pDR196 vector did not transport esculin inside the cell.

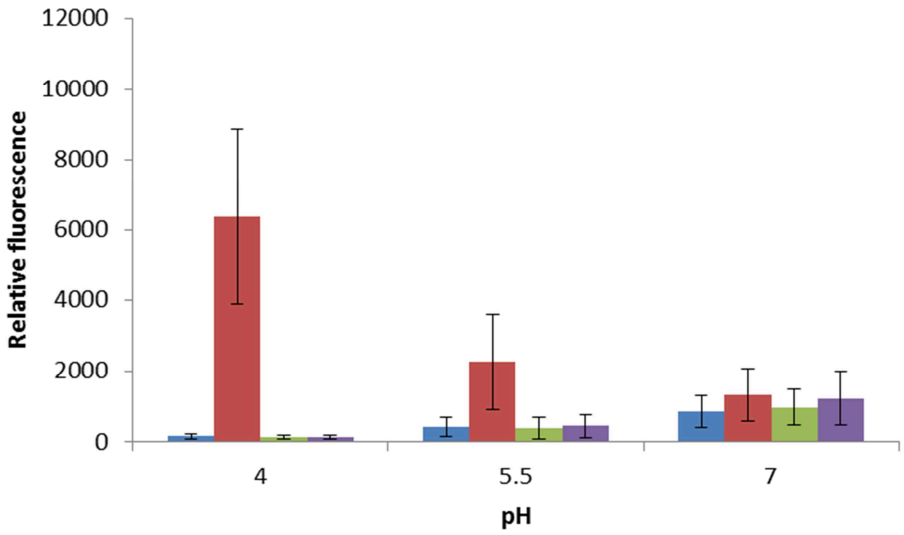


**Figure 66. Quantification of esculin uptake by yeast strain 18 GAS transformed with sucrose transporter cDNAs PpSUT1 (●), PpSUT2 (●) and PpSUT4 (●) or empty vector pDR196 (●), in three different pH conditions. Values are means of three replicates of fluorescence emission normalized per unit $OD_{600}$. Bars indicate ± SD.**

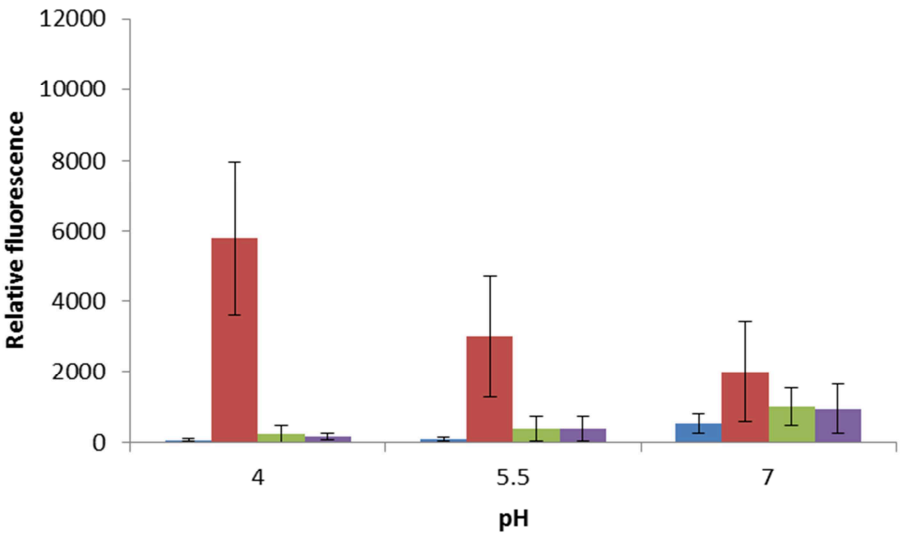


**Figure 67. Quantification of esculin uptake by yeast strain EBY.SL transformed with sucrose transporter cDNAs PpSUT1 (●), PpSUT2 (●) and PpSUT4 (●) or empty vector pDR196 (●), in three different pH conditions. Values are means of three replicates of fluorescence emission normalized per unit $OD_{600}$. Bars indicate ± SD**

### 4.3.2. Subcellular localization

The intracellular localization is an important aspect that determines the role of sucrose transporters in carbohydrates partitioning. In different plant species, the SUT proteins have been localized in two types of membranes, the plasma membrane and the tonoplast, and distinct functions have been assigned according to the membrane targeting. For these reasons, the subcellular localization of sucrose transporters from *Prunus persica* was studied by means of a transient transformation of fluorescent PpSUT fusion proteins in *Nicotiana benthamiana* leaves. To better understand of the intracellular localization, they were co-expressed with two fluorescent marker proteins, a plasma membrane marker (CBL1-OFP), and a vacuolar marker (PTR2-YFP).

**Subcellular localization of PpSUT1**

The co-expression of PpSUT1-CFP fusion protein, and PTR2-YFP vacuolar marker is shown in Figure 68 to Figure 71. The PTR2 peptide transporter was specifically localized at the tonoplast (Komarova *et al*., 2012). In mature vegetative cells, most of the volume is occupied by the vacuole, and the cytoplasm represents only a small portion of the cell, confined to periphery. Therefore, the plasma membrane and the tonoplast are often very close, but in some areas of the cell, in the proximity of the cytoplasm, can be easily distinguished. The over-expression of PTR2 YFP-fusion protein allowed the identification of the tonoplast in the epidermal cell of leaf (recognized by the

typical puzzle shape) as shown in Figure 68B. The arrows indicate the peripheral cell regions, where the cytoplasm is confined, bounded by the membrane of the vacuole. The analysis of the blue fluorescence emitted by PpSUT1-CFP (Figure 68A) pointed out that the two proteins did not co-localize. There are several evidences that support this conclusion. Firstly, PpSUT1-CFP did not label the tonoplast membrane surrounding the cytoplasm, dyed by the vacuolar marker, as highlighted by the arrows in Figure 68C, representing a merge picture between the fluorescence signals of PpSUT1-CFP and PTR2-YFP. In fact, the yellow color of tonoplast labeled by PTR2-YFP was unchanged. This evidence can be more clearly observed at higher magnification (Figure 69).

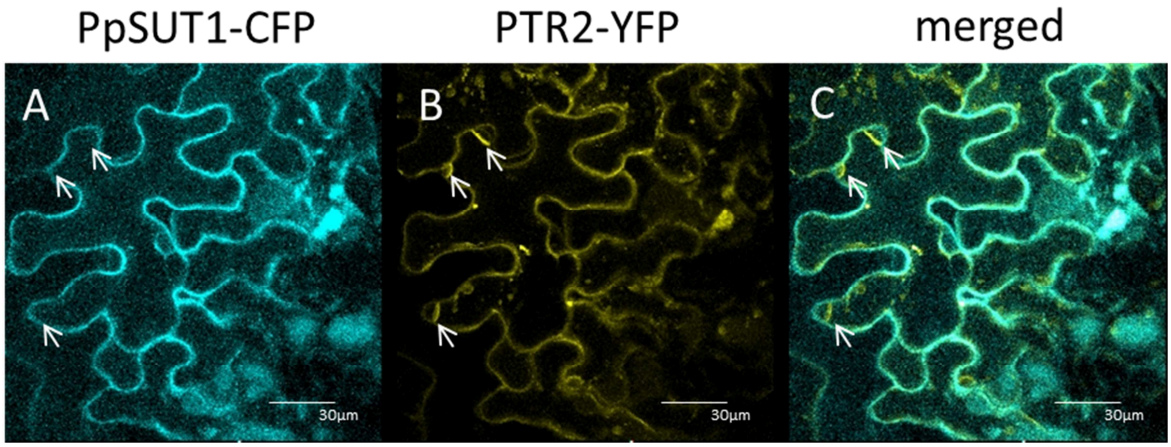


**Figure 68. Co-expression of PpSUT1-CFP (A) and vacuolar marker PTR2-YFP (B) in transient transformed *Nicotiana benthamiana* leaves. C: merged picture of A and B. Images were taken by confocal microscope and represent a maximum projection of z-stacks.**

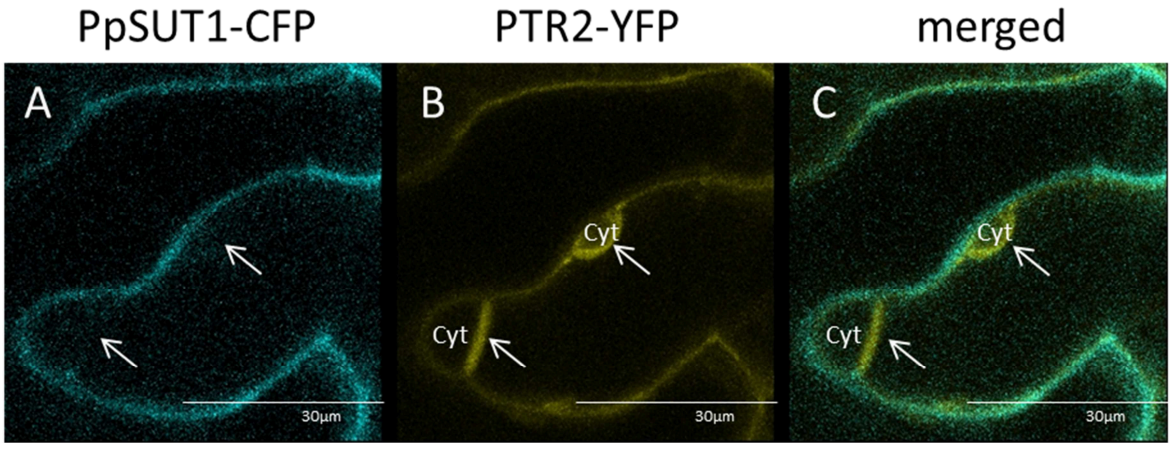


**Figure 69. Magnification of a epidermal leaf cell of *Nicotiana benthamiana* co-expressing PpSUT1-CFP (A) and vacuolar marker PTR2-YFP (B). C: merged picture of A and B; cyt: cytoplasm. Images were taken by confocal microscope and represent a maximum projection of z-stacks.**

The second clue confirming the lack of co-localization of PpSUT1-CFP and the vacuolar marker derives from the observation of two transformed adjacent cells. In this case (Figure 70B), the plasma membrane and the tonoplast were very easily distinguishable. The unstained region

between the two vacuolar membranes is likely occupied by the plasma membrane of the cells. In effect the region was labeled by PpSUT1-CFP, as evident in the overlapping of Figure 70A and Figure 70B (Figure 70C). In the cell1, the nucleus surrounded by the tonoplast, and slightly marked by the blue fluorescence of PpSUT1-CFP, was also evident.

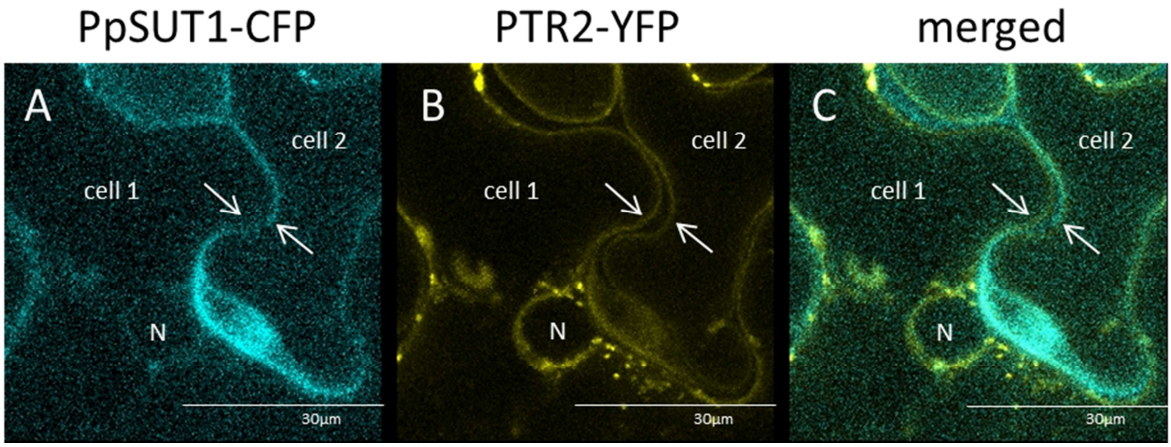


**Figure 70. Co-expression in two adjacent cells (cell 1 and cell 2) of PpSUT1-CFP (A) and vacuolar marker PTR2-YFP (B) in transient transformed *Nicotiana benthamiana* leaves. C: merged picture of A and B. N indicates the nucleus. The arrows indicate the tonoplast located in cell1 and cell2. Images were taken by confocal microscope and represent a maximum projection of z-stacks.**

Furthermore, in some cells it was possible to detect small vesicles stained by PTR2-YFP (arrows in Figure 71B) identified as small vacuoles, but not stained by the blue signal of PpSUT1-CFP (Figure 71C).

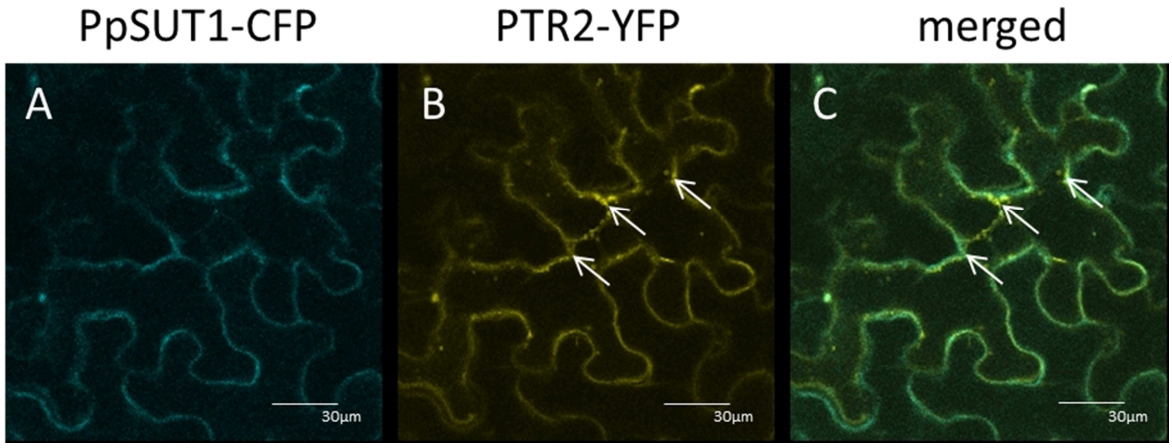


**Figure 71. Identification of small vacuole in co-transformed cells of *Nicotiana benthamiana* leaves. A: PpSUT1-CFP; B: PTR2-YFP; C: merged picture of A and B. Images were taken by confocal microscope and represent a maximum projection of z-stacks.**

On the other hand, the expression of PpSUT1-CFP and CBL1-OFP in the same cells leads to a co-localization of the two signals. CBL1 is a calcineurin B-Like protein, proved to be localized to the plasma membrane (Batistič *et al*., 2008). In fact, its signal was detected exclusively at the

periphery of the cell (Figure 72B). The blue fluorescence emitted by PpSUT1-CFP (Figure 72A) showed a behavior very similar to those of the plasma membrane marker. The overlapping of the two images indicated a co-localization of the two fusion proteins (represented by the white color) (Figure 72C). The localization on the same membrane was more evident at higher magnification (Figure 73).

In conclusion, the co-localization of PpSUT1-CFP with a plasma membrane marker, and the lack of co-localization with a vacuolar marker demonstrated that PpSUT1 was targeted to plasma membrane when transiently expressed in *Nicotiana benthamiana* leaves.

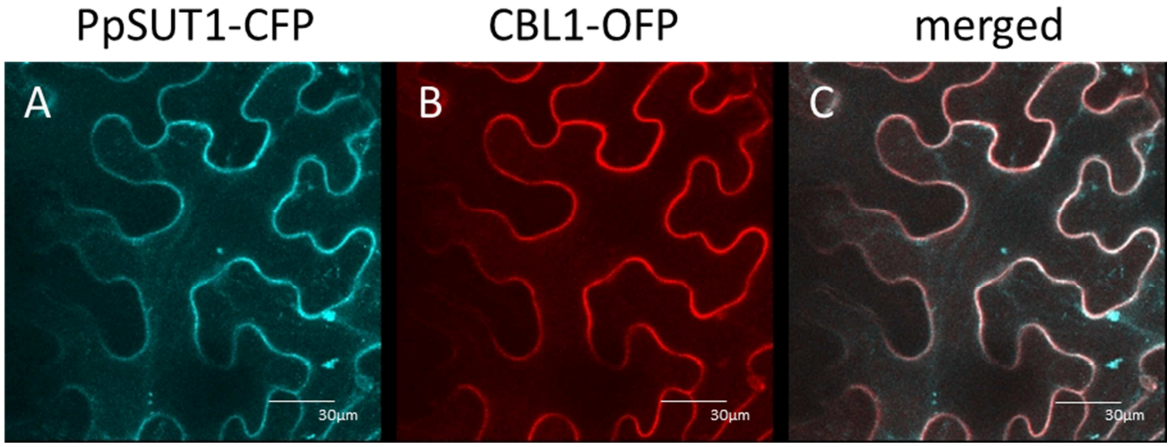


**Figure 72. Co-expression of PpSUT1-CFP (A) and plasma membrane marker CBL1-OFP (B) in transient transformed *Nicotiana benthamiana* leaves. C: merged picture of A and B. Images were taken by confocal microscope and represent a maximum projection of z-stacks.**

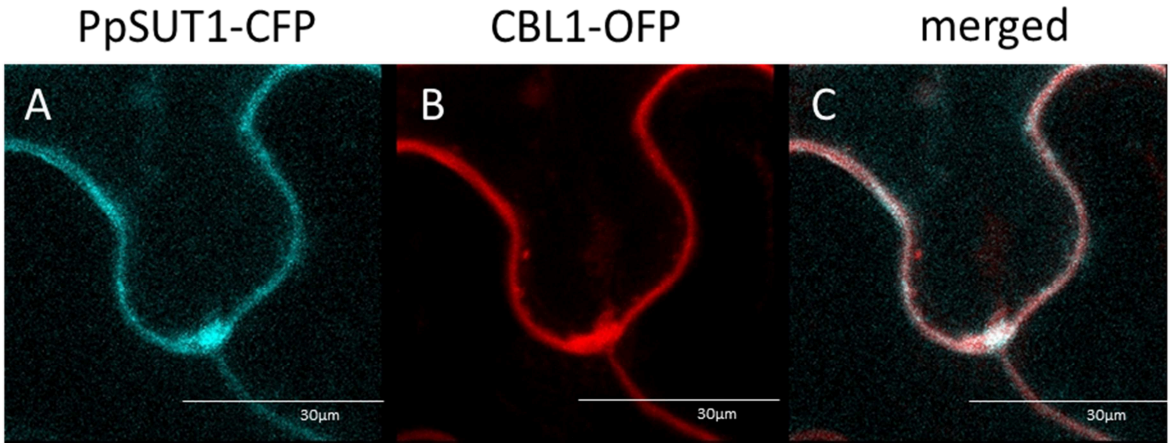


**Figure 73. Magnification of epidermal leaf cell of *Nicotiana benthamiana* co-expressing PpSUT1-CFP (A) and plasma membrane marker CBL1-OFP (B). C: merged picture of A and B. Images were taken by confocal microscope and represent a maximum projection of z-stacks.**

As the fluorescence intensity emitted by CFP is usually weaker compared to that produced by OFP or YFP, PpSUT1 was also expressed alone as YFP-fusion protein. The expression of PpSUT1-YFP allowed the identification of additional intracellular localization (Figure 74A). Indeed, the fluorescence was also evident on the membranes surrounding the nucleus, and on several structures distributed throughout the cell. These internal structures have been recognized as ER system by co-expression and co-localization of SUT proteins and ER marker (Krügel *et al*., 2008). Thus, consistent with these results, also PpSUT1 was found to localize in endomembrane.

Several researches demonstrated the redox-dependence of plasma membrane localization of sucrose transporters, as oxidizing conditions increased the efficiency of plasma membrane targeting (Liesche *et al*., 2011). In order to study this aspect, leaf discs transiently transformed with PpSUT1-YFP were incubated with reducing agent (Figure 74B). The comparison between treated and untreated sample highlighted that in reducing conditions fluorescence inside the cell increased, while that labeling the plasma membrane apparently reduced.

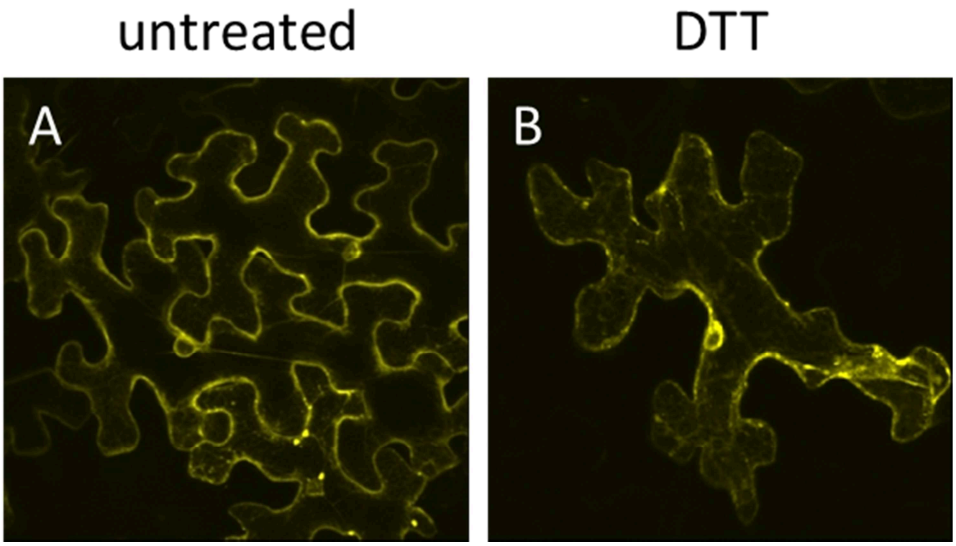


**Figure 74. A: Expression of PpSUT1-YFP fusion protein in *Nicotiana benthamiana* leaves. B: localization of PpSUT1-YFP after treatment with the reducing agent DTT.**

### Subcellular localization of PpSUT2

Cells of *Nicotiana benthamiana* leaves were also transformed with both PpSUT2-CFP and PTR2-YFP (Figure 75). The yellow fluorescence of the vacuolar marker highlighted numerous small vacuole inside leaf epidermal cell (Figure 75B). On the contrary, the blue signal emitted by PpSUT2-CFP

fusion protein (Figure 75A), appeared very weak, and this impaired the unequivocal co-localization of the two proteins on the same membrane (Figure 75C).

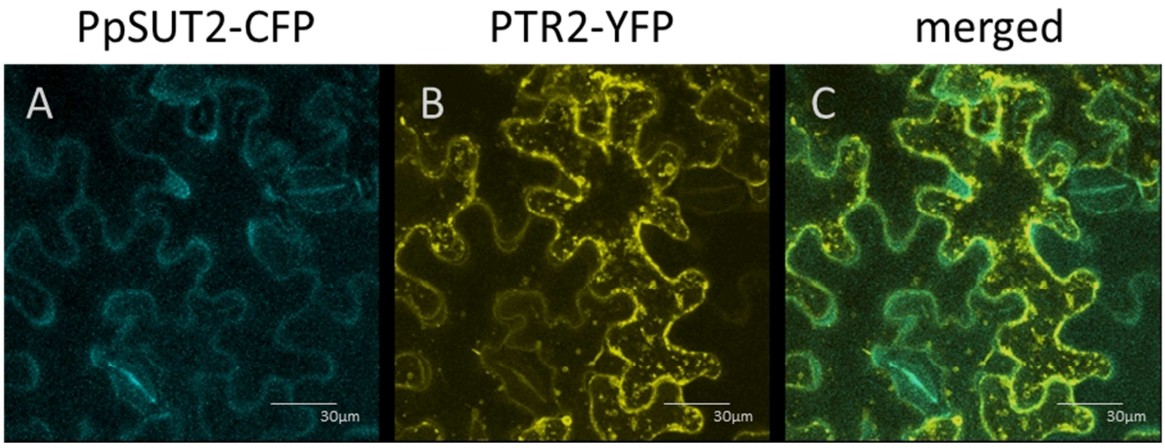


**Figure 75. Co-expression of PpSUT2-CFP (A) and vacuolar marker PTR2-YFP (B) in transient transformed *Nicotiana benthamiana* leaves. C: merged picture of A and B. Images were taken by confocal microscope and represent a maximum projection of z-stacks.**

Similar results were obtained when PpSUT2-CFP was expressed together with CBL1-OFP plasma membrane marker (Figure 76, 78).

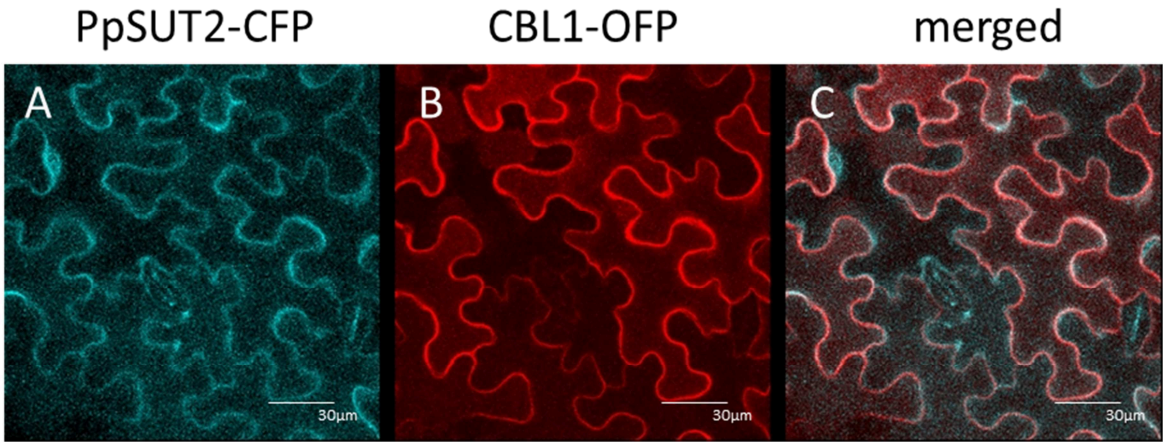


**Figure 76. Co-expression of PpSUT2-CFP (A) and plasma membrane marker CBL1-OFP (B) in transient transformed *Nicotiana benthamiana* leaves. C: merged picture of A and B. Images were taken by confocal microscope and represent a maximum projection of z-stacks.**

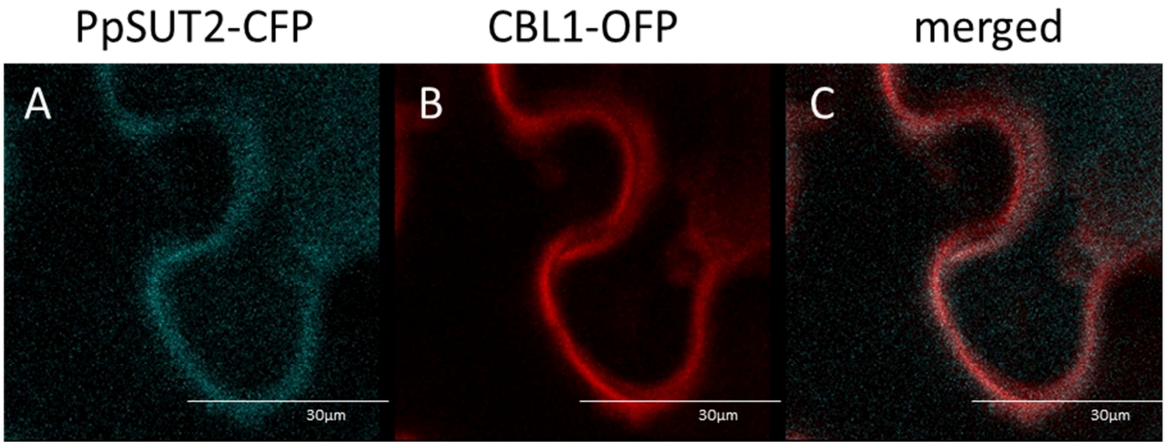


**Figure 77. Magnification of epidermal leaf cell of *Nicotiana benthamiana* co-expressing PpSUT2-CFP (A) and plasma membrane CBL1-OFP (B). C: merged picture of A and B. Images were taken by confocal microscope and represent a maximum projection of z-stacks.**

Interestingly, the expression of PpSUT2 as fusion protein with YFP revealed a specific localization on endomembrane system (ER) (Krügel *et al*., 2008). In detail, the yellow fluorescence, was mostly observed inside epidermal cells of *Nicotiana benthamiana* leaves on a membrane network of spread throughout the cell volume. The signal was also detected in the membrane surrounding the nucleus (Figure 78).

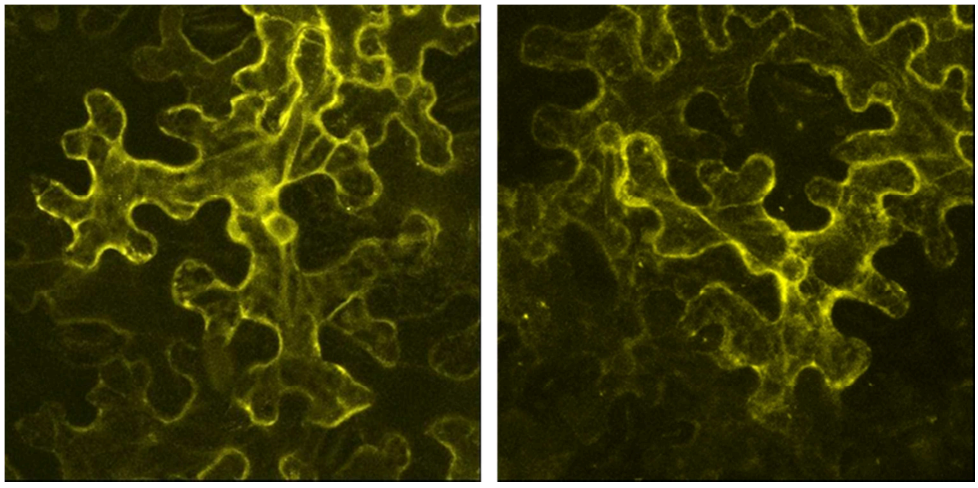

**Figure 78. Epidermal cells of *Nicotiana benthamiana* leaves expressing PpSUT2-YFP fusion protein.**

**Subcellular localization of PpSUT4**

As far as PpSUT4-CFP, the co-expression analysis with the two marker proteins suggested a localization of the sucrose transporter on the vacuolar membrane. Different members of SUT4 subfamily have already been characterized as vacuolar proteins (Chincinska *et al*., 2013). Several observation supported this hypothesis. When PpSUT4-CFP fusion protein was expressed together with the vacuolar marker the two signals localized to the same membrane (Figure 79, 81).

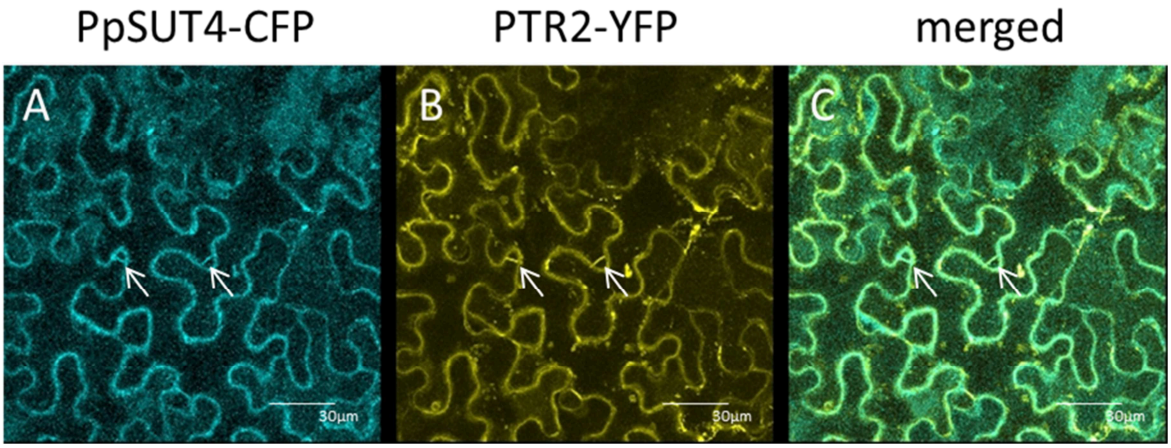


**Figure 79. Co-expression of PpSUT4-CFP (A) and vacuolar marker PTR2-YFP (B) in transient transformed *Nicotiana benthamiana* leaves. C: merged picture of A and B. The arrows point out the vacuolar membrane surrounding the cytoplasm. Images were taken by confocal microscope and represent a maximum projection of z-stacks.**

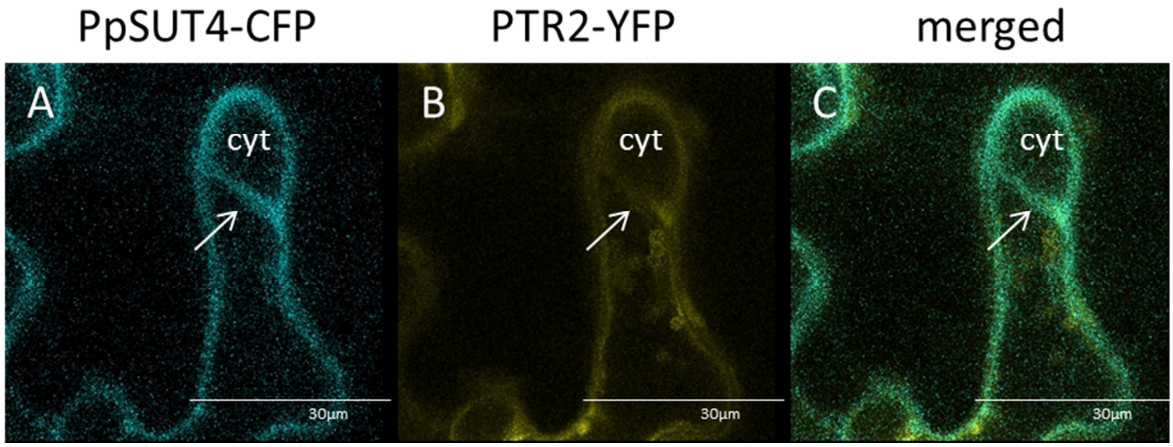


**Figure 80. Magnification of epidermal leaf cell of *Nicotiana benthamiana* co-expressing PpSUT4-CFP (A) and vacuolar marker PTR2-YFP (B). C: merged picture of A and B; cyt: cytoplasm. Images were taken by confocal microscope and represent a maximum projection of z-stacks.**

On the contrary, when the sucrose transporters were expressed together with the plasma membrane marker the two signal did not co-localized. In fact, the fluorescence emitted by

PpSUT4-CFP allowed to highlight cytoplasm-like regions, similar to those observed for the vacuolar marker, (Figure 81, 83).

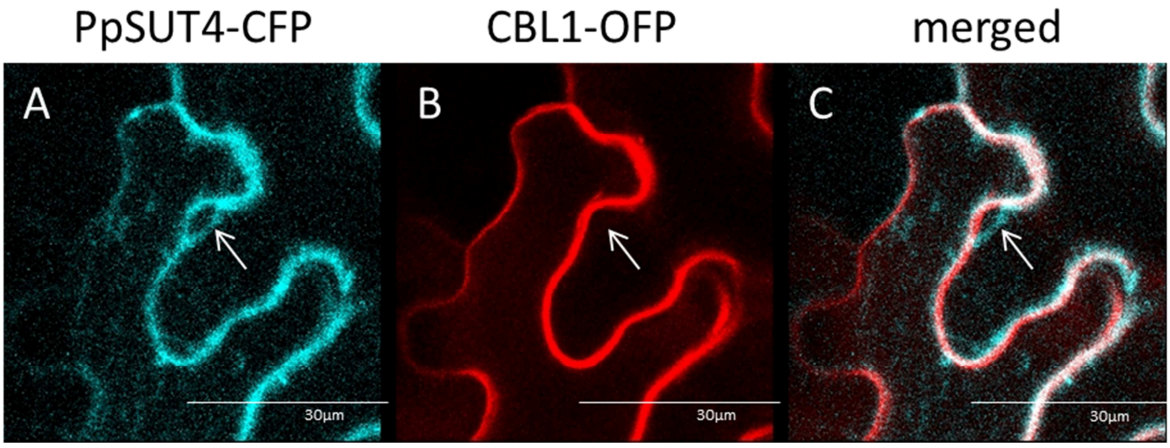


**Figure 81. Magnification of epidermal leaf cell of *Nicotiana benthamiana* co-expressing PpSUT4-CFP (A) and plasma membrane marker CBL1-OFP (B). C: merged picture of A and B; cyt: cytoplasm; arrow: tonoplast-like structure. Images were taken by confocal microscope and represent a maximum projection of z-stacks.**

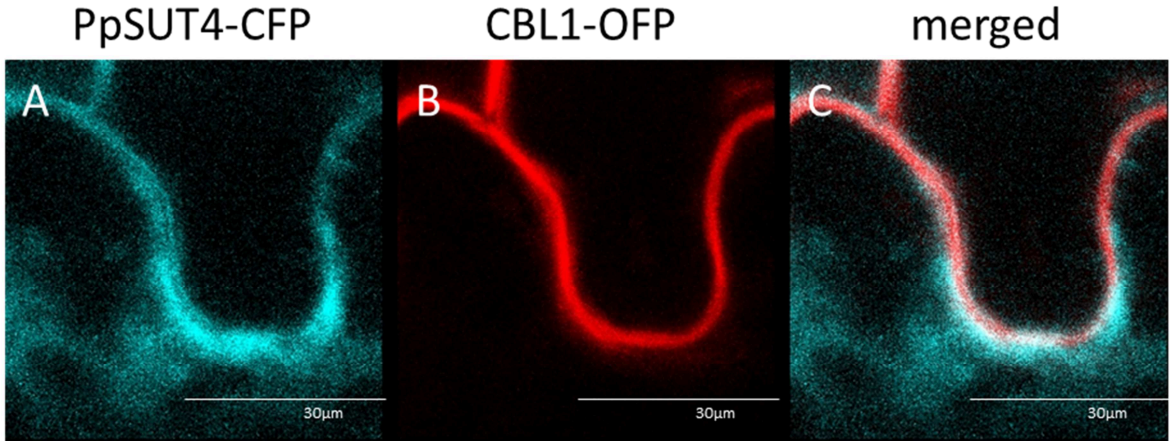


**Figure 82. Magnification of epidermal leaf cell of *Nicotiana benthamiana* co-expressing PpSUT4-CFP (A) and plasma membrane marker CBL1-OFP (B). C: merged picture of A and B; cyt: cytoplasm. Images were taken by confocal microscope and represent a maximum projection of z-stacks.**

Moreover, the expression of PpSUT4 fused to YFP revealed further information regarding the protein localization (Figure 83). The bright yellow signal of YFP was detected in the membrane surrounding the nucleus, as the other two sucrose transporters from peach, and in small vesicles. The localization in small motile vesicles has been described for StSUT1-GFP fusion protein, the size of these vesicles being about 200-500 nm, but it has been excluded that these belong to the Golgi system by co-expression with specific marker of Golgi bodies (Liesche *et al*., 2010).

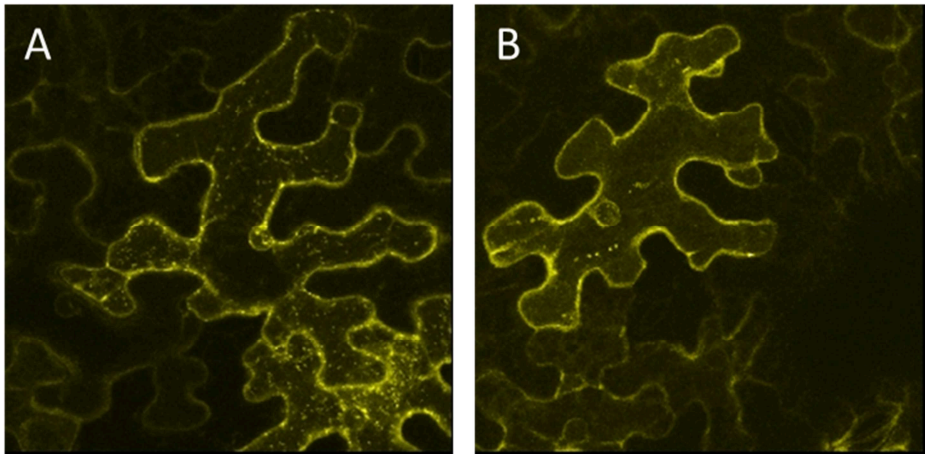


**Figure 83. Epidermal cells of *Nicotiana benthamiana* leaves expressing PpSUT4-YFP fusion protein.**

### 4.3.3. Split ubiquitin assay

The ability of sucrose transporters to interact with each other was proved in different plant species. The oligomerization has been described as one of the mechanisms that may regulate protein activity (Krügel and Kühn, 2013). To determine whether PpSUT proteins could form dimers, a split ubiquitin assay was established. The genes encoding sucrose transporters from peach were cloned as Cub fusion or Nub fusions protein. Each Cub-fusion construct was co-transformed with each Nub-fusion construct, and the ability to growth on selective medium was evaluated. The double transformants of split ubiquitin assay were grown on selective medium without histidine supplemented with 2% glucose (Figure 84). The co-expression of PpSUT1-Cub with PpSUT1-Nub, PpSUT2-Nub, and PpSUT4-Nub allowed the release of the transcription factor, activated the transcription of the reporter gene, and hence the growth of yeast cells. This indicated an interaction between the two fusion constructs. Therefore, PpSUT1 is able to form homoligomers and heteroligomers with PpSUT2, and PpSUT4 in an heterologous system. In contrast, the co-expression of PpSUT1-Nub with PpSUT2-Cub, and PpSUT4-Cub did not allow the transcription of reporter gene. This may be attributed to a difference steric hindrance of the two moieties of ubiquitin. It could be possible that the presence of the Cub, that is fused to a transcription factor, and hence presents a higher steric hindrance at the C-terminal of PpSUT2 and PpSUT4, could prevent the interaction between proteins. The expression of free Cub together with PpSUT-Nub in the cytosol did not activate HIS3, suggesting that membrane proteins interaction was required for reporter activation.

In order to evaluate the strength of the interactions between different sucrose transporters, transformants were grown in a selective medium supplemented with 2% glucose, and 40 mM 3-AT (3-Amino-1,2,4-triazole), a competitive inhibitor of the *HIS3*-gene product. In the presence of 3-AT a yeast cell, which depends upon a plasmid containing *HIS3* gene to produce histidine, have to express HIS3 gene at much higher level in order to survive. The high expression level is achieved when the interaction between bait and prey is strong. After the addition of 3-AT in the medium all transformants were able to grow in a high stringency condition, even at low cell concentration, indicating a strong protein-protein interaction (Figure 85).

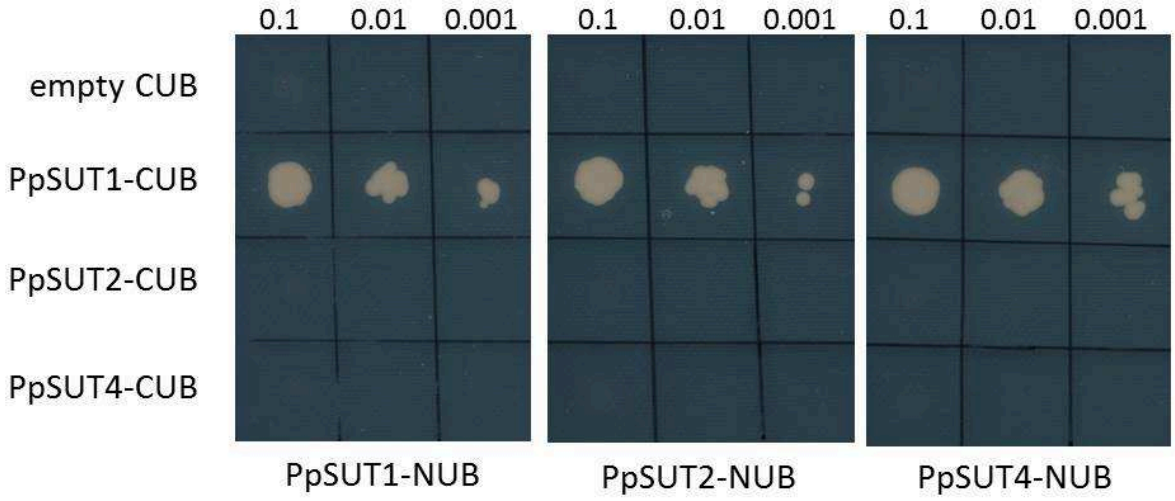


**Figure 84. Analysis of protein-protein interactions between different sucrose transporters from peach by split-ubiquitin assay. Each transformant were grown in three concentrations (0.1, 0.01 and 0.01) in a selective medium containing 2% glucose.**

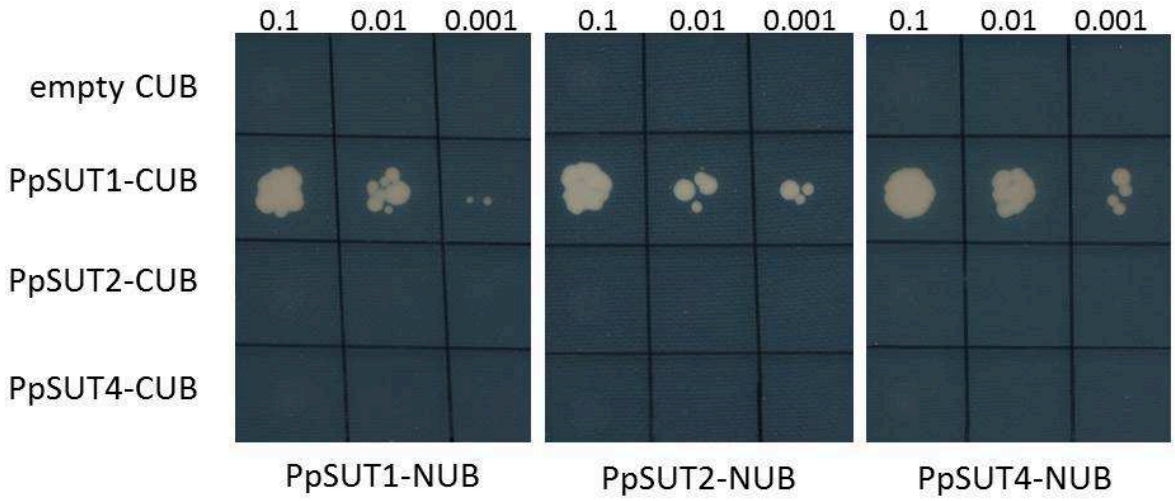


**Figure 85. Analysis of protein-protein interactions between different sucrose transporters from peach by split-ubiquitin assay in a high stringency conditions. Each transformant were grown in three concentrations (0.1, 0.01 and 0.01) in a selective medium containing 2% glucose**

With the aim of evaluate whether interaction between proteins can be influenced by the presence of substrate, all transformants were grown on selective medium supplemented with 2% sucrose. The addition of sucrose into the medium leads to a reduction of yeast growth indicating that the sugar could affect and regulate the interaction between sucrose transporters (Figure 86). However, the interaction between PpSUT1-Cub and PpSUT4-Nub appeared stronger compared to the other two transformants, this result was also confirmed in a high stringency conditions, in the presence of 3-AT (Figure 87).

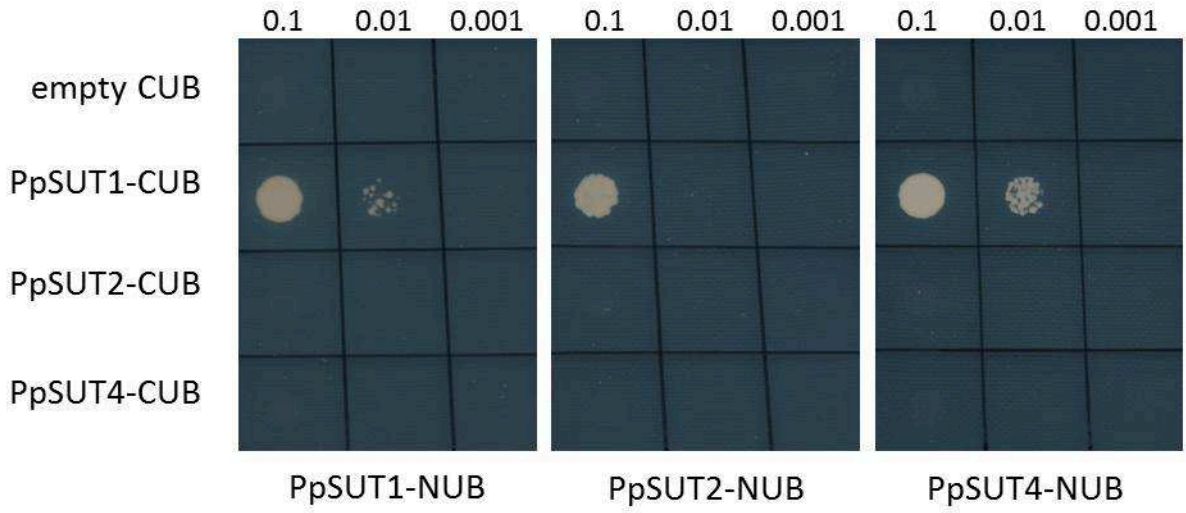


**Figure 86. Analysis of protein-protein interactions between different sucrose transporters from peach by split-ubiquitin assay. Each transformant were grown in three concentrations (0.1, 0.01 and 0.01) in a selective medium containing 2% sucrose.**

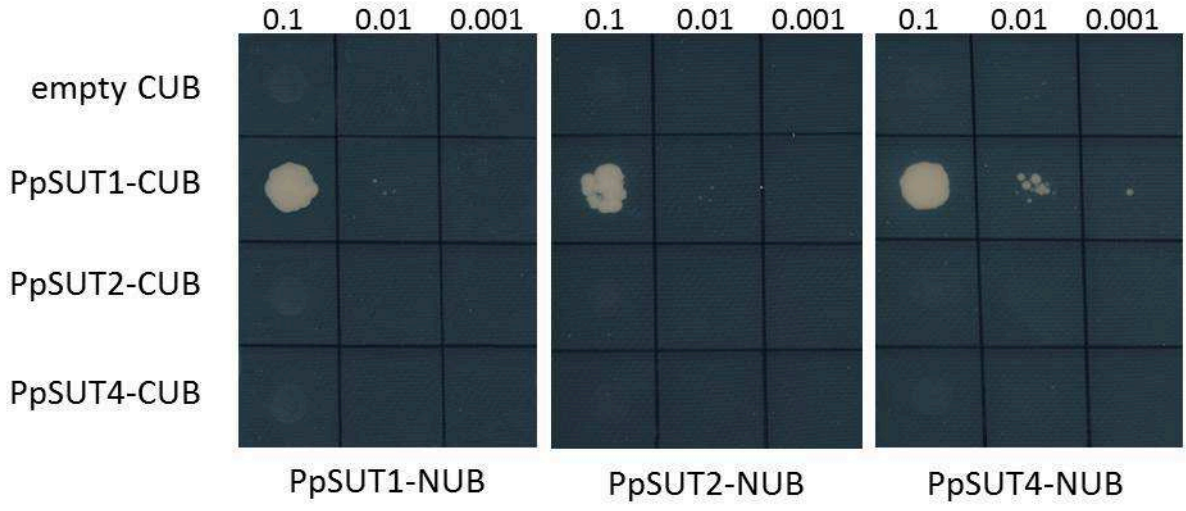


**Figure 87. Analysis of protein-protein interactions between different sucrose transporters from peach by split-ubiquitin assay in a high stringency conditions. Each transformant were grown in three concentrations (0.1, 0.01 and 0.01) in a selective medium containing 2% sucrose.**

### 4.3.4. Western-Blot analysis

After the demonstration that PpSUT1 could form homoligomers and heteroligomers in heterologous systems, the capability of the sucrose transporters to form dimer also in plant was evaluated. To this aim, a Western-blot analysis was performed on protein extracted from *Nicotiana benthamiana* leaves transiently transformed with PpSUT1-YFP fusion protein, both in the presence or absence of reducing condition. Immunodetection was performed with antibody anti-GFP able to recognized YFP tag. The antibody recognized a complex of about 75 KDa, that could represent the monomeric form of PpSUT1 fused to YFP (Figure 88). The monomeric form appeared as a double band, which is most likely due to post-translational modifications (phosphorylation/dephosphorylation) as already observed by Krügel *et al*., (2008). Moreover, a thin band of higher molecular weight was observed in the sample not treated with DTT, this could represent a oligomeric form of sucrose transporter, disappearing in the presence of DTT because reducing agents lead to dissociation of the dimeric protein, as already observed (Krügel *et al*., 2008).

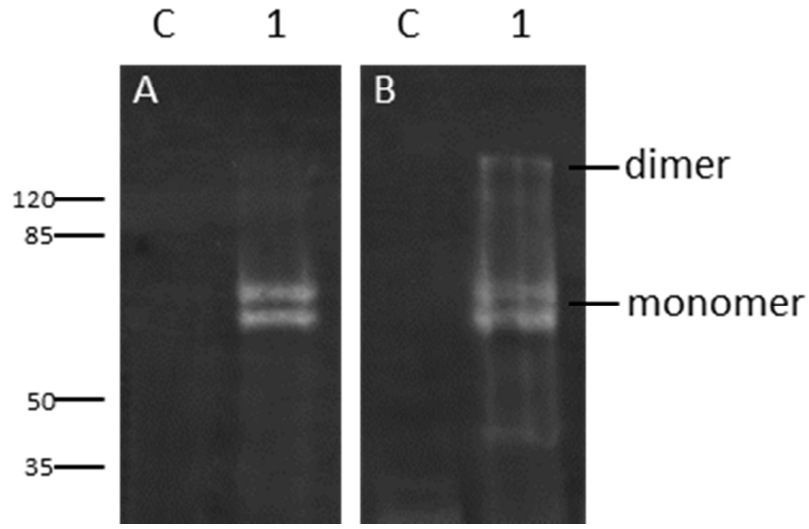


**Figure 88. Western-blot analysis of PpSUT1-YFP expression in *Nicotiana benthamiana* leaves.**

## 4.4. Discussion

Sucrose transporters (SUT) are important proteins involved in several crucial steps of long distance transport system. They contribute to move sucrose from the site of synthesis toward the site of demand. The first gene encoding sucrose transporter was discovered in spinach (*Spinacea oleracea*) and functionally characterized using a simple and useful yeast complementation strategy (Riesmeier *et al*., 1992). Since then, heterologous expression in yeast has been widely employed to analyze the function of *SUT* genes identified in several plant species. This approach was adopted, in the present work, to test the functionality of three putative genes encoding sucrose transporters, identified in the genome of peach (*Prunus persica* (L.) Batch). The genes were named *PpSUT1*, *PpSUT2* and *PpSUT4*, according to the classification of SUT proteins proposed by Kühn and Grof (2010). The three genes were expressed in two mutant yeast strains (EBY.SL and 18GAS) deficient in sucrose uptake. The functionality of both PpSUT1 and PpSUT4 was confirmed; indeed, they were able to reestablish the phenotype of mutant strains allowing the transport of sucrose inside the cells, their growth and their division in a medium containing sucrose as a unique carbon source. Moreover, the expression of sucrose transporters belonging to different subfamily within yeast cells has led to a different growth rate of the transformants. The presence of PpSUT1, allowed a more rapid growth of yeast cells compared to that of cells expressing PpSUT4, and these results are consistent with the classification in distinct subfamily. The clustering of SUT proteins in different clade, in fact, reflects not only a sequence similarity, but also similar function and substrate affinity (Kühn, 2003). Members of SUT1 subfamily have been characterized as high affinity/low capacity sucrose transporters with a Km for sucrose ranging from about 0.05 mM (AtSUC9; Sivitz *et al*., 2007) to about 1.5 mM (AtSUC2; Chandran *et al*., 2003), whereas the SUT4 clade comprise proteins with low affinity/high capacity transport with a higher Km values, for example 6 mM for StSUT4 (Kühn, 2003). Sucrose transporters from peach showed features consistent with this classification. Taken together these observation allow to hypothesize that PpSUT1 functions as a high affinity sucrose transporter, whereas PpSUT4 as a low affinity sucrose transporter. On the contrary, the functionality of PpSUT2 as a sucrose transporter was not confirmed, since the protein was unable to complement the two sucrose uptake-deficient mutant yeast strains. Some members of SUT2 subfamily, such as SlSUT2 from tomato (*Solanum lycopersicum*) (Krügel *et al*., 2013), was previously characterized as non-functional sucrose transporters, while others showed a low affinity for substrate, such as HvSUT1 from barley (Sivitz

*et al*., 2005). AtSUC3, a members of SUT2 subfamily, was initially characterized as non-functional protein (Barker *et al*., 2000), but subsequent analysis demonstrated its capability to transport sucrose (Schulze *et al*., 2000; Meyer *et al*., 2000). Due to these conflicting results, and to the characteristic structure with extended cytoplasmic domains at central loop region and at N terminus, members of the SUT2 subfamily were suggested to play a role as sucrose sensor or as regulatory protein (Barker *et al*., 2000). The failure of PpSUT2 to restore the phenotype of mutant strains might be attributed to a non-functionality of the protein, despite the possibility of an inefficient or wrong targeting of the peptide to plasma membrane in a heterologous system cannot be excluded.

Further validation of the results obtained by yeast complementation assay rose from the analysis of transformants through the 'esculin uptake system'. The treatment of the yeast cells expressing PpSUT1 with esculin confirmed the functionality of the protein. In fact, it allowed yeast to uptake esculin, making the cells fluorescent under confocal microscope; whereas both PpSUT4 and PpSUT2 were unable to transport the fluorescent dye. This result highlighted additional characteristic of PpSUT1, i.e. the capacity to recognize a different substrate. Previous works showed that members belonging to SUT1 subfamily have a specificity for substrate lower than SUT2 members, while the specificity for SUT4 group is intermediate between the two (Reinders *et al*., 2012). The capability to transport esculin has been observed only for proteins clustered into SUT1 subfamily. The specificity of peach sucrose transporters is consistent with previously findings, being only PpSUT1 able to accumulate high amount of esculin.

In addition, the quantification of fluorescent dye taken up by PpSUT1 at three distinct pH conditions (4, 5.5 and 7) demonstrated the pH-dependence of transport activity, consistent with the hypothesis of an $H^+$/symporter function.

Since the first cDNA encoding sucrose transporters discovered, the knowledge at protein level concerning substrate affinity, transport capacity, subcellular localization and activity regulation has risen. These information are important to understand the role of SUT proteins in the regulation of carbohydrate distribution, since membrane transport represents an important control point in the phloem pathway. Sucrose transport is highly regulated to optimize the partitioning towards multiple competing sink organs, and to change in relation to environmental and internal conditions (Slewinski and Braun, 2010).

An important aspect that has to be considered, to clarify the role of sucrose transporters, is the localization at subcellular level. According to this, the proteins play a role in the movement of

sucrose from the apoplast into the cell, or in the release of the disaccharide from the vacuole into the cytosol. All sucrose transporters belonging to different subfamily were localized at the plasma membrane, although SUT4 members were reported to target either the plasma membrane, or the tonoplast. Intracellular localization of plant proteins can be conveniently carried out by transient transformation of fluorescent-fusion proteins in Agrobacterium-infiltrated tobacco source leaves (Andrews and Curtis, 2005).

To understand the function of sucrose transporters from peach, PpSUT proteins were expressed as a fluorescent tag-fusion protein in *Nicotiana benthamiana* leaves, together with two distinct marker proteins (plasma membrane and vacuolar proteins).

Likewise other members of SUT1 subfamily, PpSUT1 localized to the plasma membrane, as revealed by the co-localization with plasma membrane marker, and by the lack of co-localization with vacuolar marker. Therefore, the putative function of PpSUT1 is to transfer sucrose from the apoplast, which presents pH values in the acidic range, into the cell. These results are consistent with those obtained by yeast complementation assay. Since PpSUT1 was able to complement sucrose-uptake deficient yeast cells, it had to be localized to the plasma membrane. Moreover, PpSUT1 was also detected in the endomembranes system, including those surrounding the nucleus. The dynamic localization of SUT1 proteins in different membranes was previously described, the intracellular structures being identified as endoplasmic reticulum (Liesche *et al*., 2010). Additionally, after treatment of infiltrated leaves with a reducing agent, a redox-dependence of plasma membrane targeting was observed. In fact, under reducing conditions the signal was mainly detected in endomembranes system, and in part disappeared from the cell periphery, indicating a reduction of PpSUT1 transported toward plasma membrane. A similar behavior was previously described for SlSUT1-GFP, when expressed in heterologous system (Krügel *et al*., 2008). Therefore, a modification of cytosolic redox state toward oxidizing conditions might lead to an increase of the quantity of PpSUT1 at plasma membrane, increasing sucrose uptake.

Similarly to SUT1 subfamily, members of SUT2 clade have been characterized as plasma membrane transporters. In peach, the expression of sucrose transporter belonging to SUT2 subfamily fused to a fluorescent tag failed to give clear indication about protein localization, as most of the fluorescent signal was localized within cell, in intracellular structure. Therefore, it could be possible that, after translation, the transport of PpSUT2 toward site of action was inefficient. The localization of PpSUT2 in intracellular structure might be the reason for its lack of

functionality as sucrose transporter in the heterologous system. The same behavior was previously observed in other plant species, for instance SlSUT2 from tomato (Chichinska *et al*., 2013).

In contrast, fluorescent-PpSUT4 fusion protein was localized to the tonoplast, as demonstrated by the overlap of signal with vacuolar marker, but not with plasma membrane marker. Nevertheless, PpSUT4 was able to complement a sucrose-uptake deficient yeast mutant strain, which requires at least a small fraction of sucrose transporter localized at the plasma membrane. Other proteins belonging to SUT4 subfamily, which have been assigned to the tonoplast, were able to reestablish the phenotype of yeast mutant strain, such as HvSUT2, AtSUT4 and LjSUT4 (Weise *et al.,* 2000; Reinders *et al*., 2008). This might be due to an incorrect targeting of SUT4 protein in the heterologous expression system. In addition, a recent work has highlighted a dual localization of SUT4 members either at plasma membrane, and at tonoplast according to different approaches. It could not be excluded that SUT4 proteins might target both membranes. A recent localization of Zinc-Induced Facilitator-Like 1 (ZIFL1), a transporters belonging to the MFS, has demonstrated a dual localization of the protein in relation to different alternative splicing (Remy *et al*., 2013). Sucrose transporters belonging to SUT4 subfamily might be subjected to an analogous mechanism of post-transcriptional regulation. Therefore, the localization of PpSUT4 at the tonoplast was confirmed, but further investigations are needed concerning the targeting of the protein at the plasma membrane.

In recent years, the protein-protein interaction, revealed for several membrane proteins, has been described as one of the mechanisms of post-translational regulation. The oligomerization could be essential for function, contributes to protein stability, or influences the plasma membrane targeting (Zottola *et al*., 1995; Maurel, 2007). For instance, homooligomerization of SWEETs protein in *Arabidopsis* is necessary for transport function (Xuan *et al*., 2013), while dimerization and tetramerization of mammalian glucose transporters is important for regulation of transport properties (Hebert and Carruthers, 1992). One of the main tools adopted to demonstrate the capacity of different plasma membrane peptide to form oligomers is the split ubiquitin assay (Reinders *et al*., 2002a; Schulze *et al*., 2003). The interaction between proteins in an heterologous system provides the first evidence of putative interaction also in plant.

In the present work, the split ubiquitin system allowed to identify a protein-protein interaction between peach sucrose transporters belonging to distinct subfamily. In particular, it was observed the potential capability of PpSUT1 to form homoligomers and heteroligomers with either PpSUT2 and PpSUT4 in an heterologous system. The formation of oligomeric structures might allow a

dynamic regulation of sucrose transporters. The ability of peach sucrose transporters to form oligomers in *planta* was observed at least for PpSUT1. In fact, an antibody against GFP recognized a complex with a molecular weight apparently double then monomeric form, that might correspond to the dimeric form of the transporter. Additionally, the presence of reducing agent led to a disappearance of the band, indicating a redox-dependence of proteins interaction. Oxidizing conditions might promote the protein-protein interaction of PpSUT1, that, in the dimeric form, might be targeted more efficiently to the plasma membrane, as postulated in other plant species (Krugel *et al*., 2008). In addition, the monomeric form appeared as a double band on SDS-PAGE corresponding to a different phosphorylation state of the protein, as hypothesized for other sucrose transporters (Krügel *et al*., 2008). The phosphorylation of SUT proteins may be a further mechanism of post-translational regulation.

The split ubiquitin assay highlighted the capacity of PpSUT2 to form heteroligomers with PpSUT1 in heterologous system. Since PpSUT1 was localized to plasma membrane in yeast, at least a small fraction of PpSUT2 had to be target to the plasma membrane as well, in order to reconstitute the ubiquitin and allow the release of the transcription factor for the activation of the reporter gene. Therefore the results of split ubiquitin assay provide an indirect evidence of PpSUT2 localization at the yeast plasma membrane. Consequently the inability of PpSUT2 to reestablish the phenotype of sucrose-uptake deficient mutant yeast strain could be determined by its non-functionality. The interaction between the two transporters in yeast argues for a putative interaction in *planta*, because both PpSUT1 and PpSUT2 fluorescent fusion protein were localized in endomembrane system. A sensor/signaling and regulatory role for SUT2/SUC3-type transporters were previously postulated (Barker *et al.*, 2000). Therefore, a role for PpSUT2 in the post-translational regulation of PpSUT1 may be postulated .

The hetero-oligomerization was also observed between PpSUT4 and PpSUT1. The split ubiquitin assay allowed to validate the hypothesis of a plasma membrane localization of PpSUT4 in yeast, as previously suggested by yeast complementation assay. On the other hand, the oligomerization of PpSUT4 in *planta* has to be confirmed. The two proteins, in fact, were localized in distinct biological membranes in epidermal cell of *Nicotiana benthamiana* leaves, in particular PpSUT1 to plasma membrane, and PpSUT4 to tonoplast, but both were also detected in endomembranes.

In addition, sucrose might negatively regulate the oligomerization between SUT proteins. The role of sucrose as a signaling molecule for the coordination between photosynthesis and energy demand was previously assumed (Hanson and Smeekens, 2009). It might be possible that high

level of cytosolic sucrose reduces the interaction between sucrose transporters in order to regulate the amount of sucrose uptake.

In conclusion, this work demonstrated the functionality of PpSUT1 as a sucrose transporter at the plasma membrane, and identified different types of post translational regulation (oligomerization, phosphorylation, redox regulation) that may be adopted to rapidly regulate the amount of sucrose uptake into the cell. Additionally, a role in sucrose efflux from the storage compartment was attributed to PpSUT4, as it was localized at the tonoplast. Analysis of functionality confirmed its ability to transport sucrose across biological membrane. However, further researches are needed to investigate whether it can be subjected to a dual targeting in plant cell, because members of SUT4 subfamily were localized in both plasma membrane, and tonoplast (Chincinska *et al*., 2013) and if the oligomerization might regulate its activity. Finally, the role of PpSUT2 is still unclear, as its functionality was not validated. It doesn't appear to participate to the distribution of sucrose between different compartments. However, it might have a role in the regulation of the activity of the other sucrose transporters, as hypothesized by other authors (Barker *et al.*, 2000).

# 5. Overall conclusions

Sink organs of different plant species, such as tubers, seeds and fruits, have a significant economic value, as important part of human diet. An efficient allocation of fixed carbon has a direct impact on crop production. The control of carbohydrates distribution in plants is crucial to optimize growth and development of all organs in response to internal and environmental signals. The mechanism of long distance transport has been extensively investigated, and several points of regulation have been identified along the entire pathway, that are photosynthesis, synthesis of sugars, loading, translocation, unloading, post-phloem transport, metabolic conversion and compartmentation. In sink organs, sugar distribution and metabolic conversion play an important role in sink strength (Yamaki, 2010).

In fruits sugars, besides being energy source for growth and development, are also an important trait of fruit quality. In particular, several studies focused on the key enzymes regulating metabolic conversion, but in the recent 20 years, attention has been directed to the analysis of sugar transport. Researches pointed out that fruit of different plant species utilized distinct mechanisms to import assimilates.

Peach has an important economic value, as one of the major fruit crop spread worldwide (Byrne *et al*., 2012). Due to its importance, a great number of information concerning different aspects of productivity is available. For instance, it is widely recognized that embryogenesis in peach is necessary to stimulate pericarp development, therefore crop yield is strictly connected to the efficiency of fertilization. Fruit and seed grow synchronously, displaying specific phases during development. Fruit growth can be describe as a double sigmoid. Concomitantly to the phase of growth slowdown, endocarp lignifies and embryo shows an intense mitotic activity that leads to reach its final dimension. Both mesocarp and seed compete with each other for the resources transported through the phloem system (Bonghi *et al*., 2011; Falchi *et al*., 2013). In both organs, assimilates are utilized to sustain growth and development, but they are also stored.

During peach fruit development, sugars are differently accumulated. The hexoses (glucose and fructose) predominate in young fruit, while sucrose accumulates in mature fruit (Vizzotto *et al*., 1996; Falchi *et al*., 2013). Several works have analyzed the metabolic conversion regulating their accumulation, but little is known about the mechanism of their allocation. Similarly, the assimilate transported into the seed, are used as a energy source for growth and development, and as a

substrate for the synthesis of several storage compounds, accumulated in the cotyledons in order to sustain growth of seedling during germination (Weber *et al*., 1997).

Aim of this work was to clarify the mechanism of sugars distribution in fruit and seed of peach, as important treat influencing, together with metabolic conversion, the sink strength. Analysis of CF distribution allowed to clarify, at least in part, the mechanism adopted for phloem unloading, in both mesocarp and seed. As regards to fruit, similarly to other species, the route of photoassimilates release appears developmentally regulated, changing from apoplasmic to symplasmic mechanism. In effect, an apoplasmic step in the early phase of development was previously postulated (Vizzotto *et al*., 1996). The symplasmic pathway has been associated with lower resistance and greater transport capacity as compared with apoplasmic unloading (Patrick, 1997; Patrick and Offler, 1996). Therefore, mature fruit might adopt the symplasmic unloading as predominant route to translocate a large amount of sugars into sink cells. As the sucrose reaches cytosol, of sink cells has to be rapidly removed to maintain the concentration gradient favorable to passive diffusion. This can be achieved by metabolic conversion or compartmentation. Previous research pointed out a reduction of sucrose degradative enzymes in the final phases of fruit growth, thus determining the accumulation of the disaccharide (Vizzotto *et al*., 1996). As sucrose is not metabolized in the cytosol, the presence of an efficient sucrose $H^+$/antiporter at the tonoplast, that rapidly transfers the disaccharide into the vacuole has been proposed. On the other hand, the analysis of CF distribution into the seed revealed that phloem cells are symplasmically connected to surrounding cells by functional plasmodesmata, but, as the fluorescent tracer did not spread into filial tissues, the seed coat is apoplasmically isolated from endosperm and embryo. As CF simulate the movement of sugars, it is likely that sugars reaching the seed are released from the phloem cells and diffuse throughout tegument cells, but after they reach the internal cell layer of tegument, carriers are needed for transfer toward filial tissues.

Taken together, these evidences allowed to hypothesize that both in mesocarp and in seed the activity of transporters to mediate the transfer of sucrose across biological membrane is required. Therefore, a more detailed investigation of transporters putatively involved in the assimilates partitioning in peach organs has been suggested.

At least three genes encoding sucrose transporters are present in the genome of peach, and fall into three subfamily specific of dicotyledonous, according to the classification adopted by Kühn and Grof (2010). Members belonging to distinct clades differ in several treats, for instance

substrate affinity (high and low affinity) and subcellular localization (plasma membrane and vacuolar proteins).

In this study, the transcriptional regulation, functionality, subcellular localization, and post translational regulation of the three sucrose carriers were analyzed. Taken together, results confirmed the classification into distinct subfamily. PpSUT1 is a functional plasma membrane transporter that mediates sucrose transport with high affinity. It is able to transport other substrate, as esculin, and its activity is regulated through different mechanisms. One putative mechanism is through oligomerization. Some evidences suggested that in oxidizing conditions, PpSUT1 is present in a dimeric form and it is target to plasma membrane more efficiently. PpSUT2, appears unable to mediate the transport of sucrose, and is localized mainly in endomembranes. Therefore, this carrier is suggested to play a role as a sucrose sensor. Instead, PpSUT4 is a tonoplast protein that transports sucrose with low affinity across the membrane.

These information, together with gene expression studies, and the results obtained by analysis of carboxyfluorescein allowed to hypothesize the mechanism of sucrose distribution in mesocarp and seed of peach.

In the mesocarp, during the early and middle stage of development, phloem transport occurs through an apoplasmic route, as demonstrated by the lack of CF diffusion from the phloem cells. The perception of carbohydrate availability might be mediated by PpSUT2, which expression has been detected, by *in situ* hybridization analysis, in specific cells of phloem tissue. Once in the apoplast, sucrose may be directly uptaken by sucrose transporter, or hydrolyzed and enter the cell as hexose. SUT proteins seem not involved in sucrose uptake from apoplast into sink cells, but can be mediated by another proteins, for instance, it has been shown that SWEET proteins, initially characterized as glucose uniporters, are also involved in sucrose transport (Chen *et al*., 2012). During the final phases of fruit development, there is a shift in the mechanism of phloem unloading, with the involvement of a symplasmic step, as demonstrated by the release of CF from phloem bundles. At this stage, sucrose moves from phloem cells to sink cells through plasmodesmata and accumulates. The storage of sucrose into the vacuole could be, in effect, determined by the down-regulation of Ppsut4, that inhibits the efflux of sucrose from the vacuole. On the other hand, assimilates compartmentation, during the last stages of development is consistent with the need of a gradient driving an efficient phloem unloading.

In the seed, a different role for the three PpSUT sucrose transporters has been proposed. Its development is characterized by a temporary storage of sugars in the endosperm during the early

phase of development, and a re-allocation of carbohydrates from endosperm into the cotyledons during the rapid growth of embryo. Sucrose transporters might participate to sugar distribution in different manner. PpSUT1 which is mainly expressed in early seed development might contribute to determine endosperm growth and development, mediating the uptake of sucrose from the apoplast into endosperm cells. Besides, PpSUT2 and PpSUT4 may have a role in the re-allocation of sucrose between endosperm and embryo, during the phase of endosperm reabsorption, as their transcripts localized in the interface between the two tissues. Finally, PpSUT4 might have an additional function during the maturation phase of seed, mediating the efflux of sucrose from the vacuole, and supplying substrates for the synthesis of storage compounds.

This research represents the first attempt to clarify the pathway of assimilates distribution in developing peach fruits. In order to explain the steps of active transport involved in these mechanism, particular attention has been paid to carriers, providing an extensive characterization of all sucrose transporters identified in peach genome. All evidences, deriving from both histological and molecular studies, allowed to propose an hypothesis concerning the role of the sucrose transporter in carbohydrates partitioning, even if the involvement of other proteins has been suggested in order to completely resolve this complex mechanism.

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Part of the research activity was focused on other topics, represented by the following publications:

Falchi R., Vendramin E., Zanon L., Scalabrin S., Cipriani G., Verde I., Vizzotto G. and Morgante M. (2013). Three distinct mutational mechanisms acting on a single gene underpin the origin of yellow flesh in peach. The Plant Journal 76(2):175-87.

Falchi R., Zanon L., De Marco F., Nonis A., Pfeiffer A. and Vizzotto G. (2013). Tissue-specific and developmental expression pattern of abscisic acid biosynthetic genes in peach fruit: possible role of the hormone in the coordinated growth of seed and mesocarp. Journal of Plant Growth Regulator 32(3): 519-532.

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